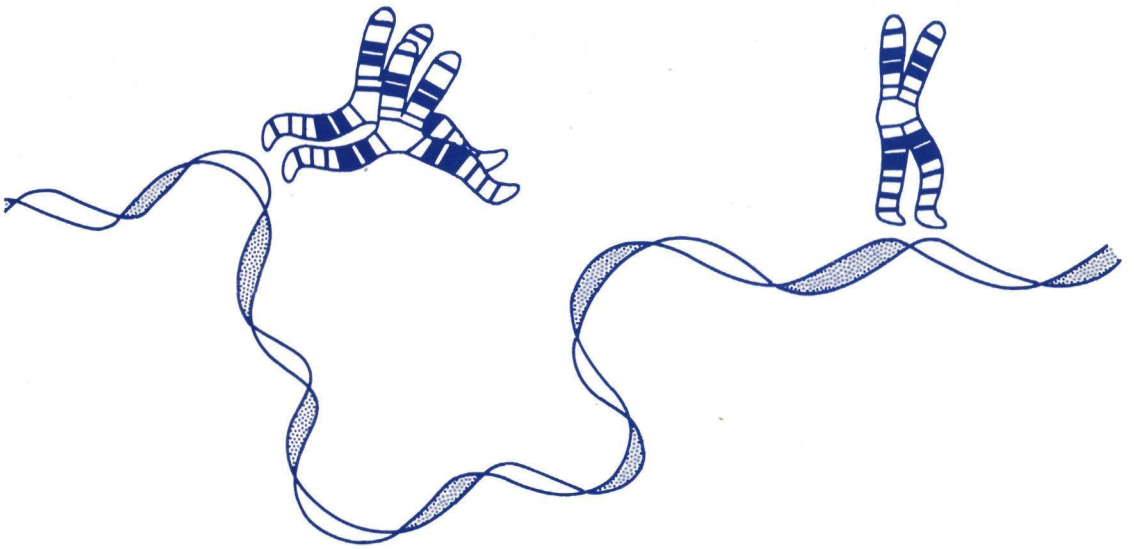
POSITIONAL CLONING OF A CANDIDATE GENE FOR CHOROIDEREMIA



F.P.M. Cremers

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Het onderzoek voor dit proefschrift werd gesubsidieerd door de Nederlandse Organisatie voor Wetenschappelijk Onderzoek, de 'Deutsche Forschungsgemeinschaft', de Landelijke Stichting voor Blinden en Slechtzienden, de Gelderse Blinden Vereniging, de stichting Blinden-Penning, en de 'British Retinitis Pigmentosa Society.'

Publikatie van dit proefschrift is mede mogelijk gemaakt door giften van WESTBURG B.V. en GIBCO BRL.

POSITIONAL CLONING OF A CANDIDATE GENE FOR CHOROIDEREMIA

een wetenschappelijke proeve op het gebied van de geneeskunde en tandheelkunde, in het bijzonder de geneeskunde

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Katholieke Universiteit te Nijmegen volgens besluit van het college van decanen in het openbaar te verdedigen op donderdag 18 april 1991 des namiddags 3.30 uur

door

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geboren op 17 maart 1960 te Jabeek

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Prof. Dr. B. Wieringa

Voor mijn ouders

Voor Diana, Ruud & Theo

Dankwoord

Dit proefschrift zou niet tot stand zijn gekomen zonder de steun van mijn vrouw Diana, die zowel pieken als dalen tijdens het onderzoek steeds weer wist te relativeren.
Mijn ouders ben ik veel dank verschuldigd, omdat zij mij de mogelijkheid hebben gegeven om te gaan studeren. Tijdens mijn studie Biologie werd de keuze voor moleculaire biologie voor een groot deel bepaald door de boeiende colleges van John Schoenmakers. In zijn laboratorium heb ik de basistechnieken op een uitstekende manier geleerd van Roselie Jongbloed en Johan den Dunnen.
Paul Reekers wil ik danken voor het feit dat hij mij de mogelijkheid heeft geboden de eerste zelfstandige schreden te zetten op het onderzoekerspad. Door de steun die ik van Willem Raaben, Henk Tijssen, Jos Ruiter, en Mia van de Beuken van de Universitaire Transfusie Dienst (UTD) kreeg, bewaar ik goede herinneringen aan deze tijd.
Hilger Ropers dank ik voor het vertrouwen dat hij in mij gesteld heeft, voor zijn deskundige begeleiding bij het schrijven van publikaties, en voor zijn positieve benadering van alles wat met het TCD onderzoek van doen had.
Bé Wieringa dank ik voor zijn deskundige en enthousiaste begeleiding gedurende zowel de 'UTD-tijd' als tijdens het promotie-onderzoek.
Het opsporen van genen betrokken bij erfelijke ziektes vereist samenwerking met vele tientallen onderzoekers uit de hele wereld, welke ik hier niet allemaal kan noemen. De basis van ons onderzoek werd gelegd door Marten Hofker van het Sylvius laboratorium te Leiden die de meeste DNA markers heeft geleverd. Ik wil hierbij ook Lau Blonden bedanken die zeer behulpzaam was bij het identificeren van cosmid-clonen.
I am especially grateful to Frank Brunsmann and Eeva Sankila, who not only supported us by collecting TCD patient material, but also became good friends.
De grootste bijdrage aan dit proefschrift werd geleverd door Dorien van de Pol, wiens inzet en nauwgezette manier van werken van zeer grote waarde zijn geweest. Belangrijke bijdragen werden ook geleverd door Liesbeth van de Kerkhoff, Karen Molloy, Frank Oerlemans, Liesbeth Boender-van Rossum, Wolfgang Berger, Paul Diergaarde, en de studenten Richard Brohet en Francy van de Klundert. Zij allen hebben de goede sfeer binnen de 'TCD-groep' bepaald.
Verder ben ik dank verschuldigd aan Jos Domen, Jack Leunissen, en Celia van Gelder voor de analysen van cDNA clones.
Alle niet met naam genoemde collega's van het Anthropogenetisch Instituut wil ik bedanken voor de goede sfeer en samenwerking welke sterk heeft bijgedragen tot dit resultaat.

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Chapter 1

General Introduction

1.1 The human genome

1.1.1 Organization

The haploid human genome comprises approximately 3 billion base pairs (bp) of DNA and is estimated to contain 50,000-100,000 genes. In diploid cells, the nuclear genes are distributed over 22 pairs of autosomes in addition to two X-chromosomes in females and one X and one Y chromosome in males. The mitochondrial chromosome has a length of 16,569 bp, and is present in thousands of copies (approximately 10 per mitochondrion) in a diploid cell. Most genes located on nuclear chromosomes are interrupted by introns and are separated by long non-coding segments of DNA. Therefore, only about 2-5% of the DNA sequence is transcribed into mature RNAs. On the basis of reassociation kinetics, it has been estimated that approximately 60% of the nuclear genome is comprised of repeated sequence DNA. In contrast, the mitochondrial chromosome has a very high density of coding information; there are no introns, and there is very little non-coding DNA between genes (Anderson et al. 1981).

1.1.2 DNA sequence variation

While in general, DNA sequences that code for proteins are highly invariant, a remarkable degree of polymorphism has been found in non-coding single-copy sequences. The first DNA polymorphism of this kind was discovered in the 5′ flanking region of the β-globin gene by Kan and Dozy (1978). Association of one of the marker alleles with the defect underlying sickle cell anaemia permitted, in an indirect way, the diagnosis of this disorder without testing for the sickle cell mutation itself (Little et al. 1980). In the early eighties a large number of anonymous DNA fragments, several of which identified RFLPs, were cloned. Such restriction fragment

length polymorphisms (RFLPs) are numerous, widespread in the genome, and segregate in a Mendelian manner. On average, the human genome shows sequence variation in every 100 to 1000 bp of DNA (Jeffreys 1979; Murray et al. 1984; Hofker et al. 1986). Jeffreys and coworkers (1985) were the first to describe a marker system in which the polymorphism is due to variation in the number of tandem repeats of a 10-15 bp DNA sequence. Because most individuals will be heterozygous at such loci, these markers provide linkage data in almost all families. They were named minisatellite or "variable number of tandem repeat" (VNTR) markers, and isolated on a large scale by Nakamura and coworkers (1987). Recently, a variant on the VNTR marker system has been found, which is based on variable numbers of simple sequence motifs (VSSMs; Litt and Luty 1989; Smeets et al. 1989; Weber and May 1989). In the human genome there are 50,000-100,000 interspersed blocks of tandemly repeated CA-dimers, which, if uniformly spaced, will provide highly polymorphic markers every 30-60 kilobases (kb)(Weber and May 1989).

1.1.3 The X chromosome

The human X chromosome, as estimated from its DNA content and its length in metaphase spreads, comprises 5-6% of the haploid genome (Mendelsohn et al. 1973; Southern 1982), which corresponds to 160,000-200,000 kb. Studies in lower vertebrates, in particular Amphibians and Reptiles, have suggested that the X and Y chromosomes have evolved from a single pair of chromosomes. Ohno (1967) proposed that the sex chromosomes have evolved differently after the occurrence of a major inversion inhibiting meiotic crossing over in the inverted region and permitting a gradual genetic divergence of the two chromosomes. Only the tips of the short arms, the so called pseudoautosomal regions, have remained genetically identical (Burgoyne 1982). This region is probably needed for chromosome pairing and chiasma formation between X and Y, which is necessary for the proper distribution of the

chromosomes to the daughter cells during meiosis. Other regions of homology probably represent more recent events of DNA exchange between the X and Y chromosomes (Page et al. 1984; Geldwerth et al. 1985).

In the course of evolution, the Y chromosome has become largely dysfunctional. No more than 6 functional loci are known to be located on the short arm, namely two cell-surface markers, MIC2Y and Yg, ZFY, a gene encoding a zinc-finger protein involved in spermatogenesis (Page et al. 1987), SRY, a candidate gene for the testis determining factor (Sinclair et al. 1990), RPS4Y, a gene encoding a ribosomal protein (Fischer et al. 1990), and a gene encoding a granulocyte-macrophage colony stimulating factor (Gough et al. 1990). In addition, other gene(s) involved in spermatogenesis have been assigned to the long arm (Tiepolo and Zuffardi 1976).

Parallel to the loss of Y-chromosomal genes, a dosage compensation mechanism evolved on the X chromosome, rendering one of the two chromosomes largely inactive in female somatic cells. Inactivation of one of the two X chromosomes takes place in all embryonic cells in a random fashion during the 4th day of development, so that both X chromosomes are only active during oögenesis and the very early embryonic development (Gartler et al. 1975). The process of X chromosome inactivation is controlled by a cis-acting locus which maps to the Xq13 band (Mattei et al. 1981; Brown et al. 1991b). Recently, a candidate gene for X-inactivation control (XIST, X inactivation specific transcript) has been identified in the same band which is only expressed on the inactive X chromosome (Brown et al. 1991a). Initially it had been assumed that only genes that map (close) to the pseudoautosomal region escape X-inactivation, such as the steroid sulphatase gene, MIC2X, and Xg. Recently however, several genes from other parts of the X chromosome have been found to remain active, too, on inactivated X chromosomes (Schneider-Gädicke et al. 1989; Brown and Willard 1990; Fischer et al. 1990). It is striking that all genes that have been shown to escape from inactivation, except for the RPS4X gene (Fischer et al. 1990),

map to the short arm of the human X chromosome. Several of these genes may represent evolutionary recent acquisitions of the X, as suggested by their autosomal location in various marsupials (or 'non-placental' mammals; Sinclair et al. 1988). The X chromosome is known to contain a minimum of 160 genes and disease loci (McKusick 1990), several of which are depicted in Figure 1. Because of their distinctive inheritance patterns, X-linked traits have been identified earlier and in relatively larger numbers than autosomal traits (McKusick 1990). The majority of X-linked diseases are only defined as clinical entities, while the underlying biochemical defects are unknown. Many of these defects have been regionally mapped

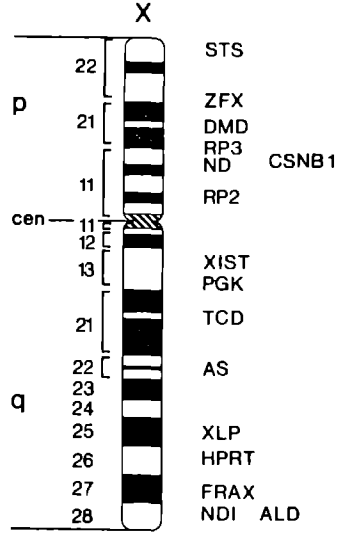

Fig. 1. Map of the X chromosome showing the position of various genes and disease loci. STS = steroid sulphatase, ZFX = X-borne zinc-finger protein, DMD = Duchenne muscular dystrophy, RP2 and RP3 = retinitis pigmentosa type 2 and type 3, ND = Norrie disease, CSNB1 = congenital stationary night blindness type 1, XIST = X inactivation specific transcript, PGK = phosphoglycerate kinase, TCD = tapetochoroidal dystrophy, AS = Alport syndrome, XLP = X-linked lymphoproliferative disease, HPRT = hypoxanthine phosphoribosyltransferase, FRAX = fragile (X) syndrome, NDI = nephrogenic diabetes insipidus, ALD = adrenoleukodystrophy

on the X through linkage studies with DNA markers. This has paved the way for the isolation of the relevant genes by positional cloning strategies (1.2).

1.2 Gene mapping and positional cloning

1.2.1 Genetic and physical mapping

Conventional biochemical approaches to the cloning of genes underlying genetic disorders require that the fundamental biochemical defect and the structure of the relevant gene product are known. For most of the known Mendelian disorders of man, however, this information is not available. This explains why, at present, only a minority of the relevant genes have been cloned (McKusick 1990). An alternative approach, termed "positional cloning" or "reverse genetics strategy", aims at the isolation of these genes on the basis of their known (sub)chromosomal location. For the chromosomal assignment of gene defects, both genetic and physical methods have been employed.

Linkage analysis makes use of large numbers of polymorphic DNA markers that have been isolated from the human genome. Approximately 150 highly informative markers, evenly spaced throughout the genome at a distance of 20 cM, are required to establish linkage (Botstein et al. 1980). For the X chromosome, approximately 10 evenly spaced informative markers suffice, in principle, to detect linkage with any given locus. Once linkage is established, additional markers can be employed to narrow down the location of the disease gene to one or a few centiMorgans (cM) from a polymorphic marker. More accurate estimation of genetic distances is seldomly possible, given the rarity of most Mendelian disorders and the limiting family sizes. 1-5 cM roughly correspond to 1,000-5,000 kb. Such a stretch of DNA is too large to be bridged by employing conventional lambda or cosmid cloning vectors, which can carry 20 and 40 kb of human DNA, respectively.

Physical mapping methods are based on the analysis of chromosomal aberrations, e.g. deletions or translocations, that are associated with recognizable genetic disorders. In this way, a far more precise localization of disease genes can be achieved. On the X-chromosome, numerous gene defects were found to be associated with interstitial deletions involving bands Xp22.3 (ichthyosis vulgaris, kallmann syndrome, chondrodysplasia punctata; Ballabio et al. 1989), Xp21 (Duchenne muscular dystrophy [DMD], chronic granulomatous disease [CGD], McLeod phenotype, and retinitis pigmentosa [RP]; Francke et al. 1985), Xp11.3-p11.4 (Norrie disease; Gal et al. 1986b), Xq21 (choroideremia, deafness, mental retardation; Rosenberg et al. 1986, Hodgson et al. 1987), and Xq25 (X-linked lymphoproliferative disease; Wyandt et al. 1989). A small number of X-linked diseases have been found to be associated with balanced X-autosomal translocations in females. Here, the clinical symptoms are due to disruption of the relevant gene and the fact that in balanced X-autosome translocations, the normal X chromosome is preferentially inactivated (Mattei et al. 1982). X-autosome translocations have facilitated the mapping, and in some cases cloning, of disease loci underlying Aicardi syndrome (Ropers et al. 1982), Aarskog syndrome (Bawle et al. 1984), choroideremia (Kaplan et al. 1989; Siu et al. 1990; this thesis), DMD (Verrellen-Dumoulin et al. 1984; Ray et al. 1985), Hunter's disease (Mossman et al. 1983), hypohidrotic ectodermal dysplasia (Gerald and Brown 1974; Zonana et al. 1988), Hypomelanosis of Ito (Hodgson et al. 1985), Lowe oculocerebrorenal syndrome (Hodgson et al. 1986), and Menkes syndrome (Kapur et al. 1987).

1.2.2 Positional cloning methods

Several methods are available to bridge the gap between genetically or physically linked markers and a disease gene. Two of these methods have been employed in this study and will be discussed briefly. For chromosome jumping, large DNA

molecules (50-500 kb) are circularized and the junction fragments containing both ends of the DNA molecule are subsequently cloned in a lambda phage or plasmid vector. In this way, it is possible to circumvent insert size constraints of conventional cloning vectors which limit the distance that can be covered by individual chromosome walking steps. When the orientation of a given starting clone and the disease locus is known, each chromosome "jump" will result in a new clone 50-500 kb closer to the disease locus (Collins and Weismann 1984; Poustka and Lehrach 1986; Collins et al. 1987; Poustka et al. 1987).

Preparative field inversion gel electrophoresis (FIGE) and pulsed field gel electrophoresis (PFGE) aim at the enrichment of large DNA fragments carrying the respective gene, preferably from a human/rodent cell hybrid containing only the human chromosome of interest. By establishing a DNA library from an enriched DNA fragment, human clones can be identified and isolated by screening with human DNA (Michiels et al. 1987; Anand et al. 1988). Following this strategy and employing new DNA markers as starting points for extensive chromosomal walking, one can ultimately obtain an overlapping set of lambda- or cosmid-clones from the critical region.

The final step involves the identification and isolation of genes in the relevant chromosome segment. For this, two approaches have been widely employed. The first one is based on the expectation that exons are more stringently conserved during evolution than non-coding sequences. Therefore, exons can be identified by searching for non-repetitive DNA segments that detect homologous sequences in DNA from other vertebrate species. According to the second strategy, probes from the critical chromosome region are hybridized to Northern blots containing RNA from tissues in which the disease gene is expected to be expressed. Definite proof for the identity of a candidate gene must come from studies demonstrating its lack or aberrant expression in patients suffering from the disease.

Chromosomal abnormalities have played a key role in positional cloning efforts which resulted in the isolation of

genes underlying retinoblastoma (Friend et al. 1986), DMD (Monaco et al. 1986), CGD (Royer-Pokora et al. 1986), Wilms tumour (Call et al. 1990; Gessler et al. 1990), colon carcinoma (DCC, deleted in colon carcinoma; Fearon et al. 1990), testis-determination (Sinclair et al. 1990), and neurofibromatosis type 1 (Cawthon et al. 1990; Viskochil et al. 1990; Wallace et al. 1990). In contrast, the regional assignment of the cystic fibrosis gene to chromosome 7q31, which formed the basis for its subsequent isolation (Kerem et al. 1989; Riordan et al. 1989; Rommens et al. 1989), was achieved exclusively through large scale RFLP linkage analysis (Tsui et al. 1985; Wainwright et al. 1985; White et al. 1985).

1.3 Chorioretinal disorders

1.3.1 Genetics of retinal dystrophies

Retinitis pigmentosa (RP) is the name commonly given to a group of hereditary diseases characterized by progressive dysfunction of the photoreceptors, which is often followed by degeneration of other retinal cell layers. Visual impairment usually becomes manifest as night blindness, visual field loss, and, in some cases, central visual dysfunction. RP encompasses not only a large number of diseases that are confined to the eye, including classical RP and choroideremia, but also disorders involving other organs or tissues (Heckenlively 1988). Classical forms of RP can be inherited in an autosomal dominant (AD), autosomal recessive (AR), or X-linked (XL) mode. Until very recently, no biochemical defects were known for any of the RP-types. Therefore, several research groups have set out to localize different forms of RP employing both physical and genetic methods aiming at the isolation of the respective genes.

McWilliam et al. (1989), employing a large Irish family, were the first to establish linkage between a type I AD-RP locus and the chromosome 3q locus D3S47 in the vicinity of the rhodopsin gene. With this knowledge at hand, Dryja and co-

workers (1990) identified a point-mutation in codon 23 of the rhodopsin gene, in 17 of 148 unrelated ADRP patients. The point-mutation results in the substitution of a proline residue which is highly conserved among the opsins and related G-protein receptors (Applebury and Hargrave 1986). Dryja's finding indicates that the rhodopsin mutation is the cause for at least part of the AD forms of RP. The fact that no mutations in the rhodopsin gene have been found in the Irish ADRP family (Farrar et al. 1990a,b), together with recent evidence that there may be a gene locus at another position of 3q, indicate that at least one, and possibly several other genes, may be involved in AD-RP (A.Gal, personal communication).

It is likely that autosomal recessive RP is also heterogeneous, but linkage analyses have so far failed to identify the location of genes that give rise to 'classical' AR-RP. In the mouse, two genes underlying recessive retinal disorders, i.e. rds (retinal degeneration slow) and rd (retinal degeneration), have been cloned and assigned to chromosomes 17 and 5, respectively (Travis et al. 1989; Bowes et al. 1990). The rds gene encodes a transmembrane protein which is located in the rod outer segment discs (Dr. G.H. Travis, personal communication). The rd gene codes for the β subunit of the rod cGMP-phosphodiesterase (Bowes et al. 1990). Homologues of these genes could also play a role in the etiology of human AR-RP. A gene underlying Usher syndrome type II which is characterized by autosomal recessive RP and hearing loss, was localized recently to chromosome 1q through demonstration of linkage to anonymous DNA markers (Kimberling et al. 1990; Lewis et al. 1990). So far, no candidate genes are known so far to lie in the vicinity of these markers. Usher syndrome type I families did not show linkage to the 1q marker indicating that this syndrome is not allelic to Usher type II.

Linkage between DXS7, a X-chromosomal marker from p11.3, and XL-RP was first established by Bhattacharya et al. (1984). Subsequent analyses strongly suggested that there are at least

two different loci for XL-RP, one gene being just distal to OTC (Xp21.1; RP3, Fig. 1), and another gene located between DXS14 and DXZ1 (Xp11.1-p11.2; RP2, Fig. 1). There is evidence for even a third XL-RP locus, between DXS28 and DXS164 at Xp21 (Ott et al. 1990). The location of an XL-RP locus at Xp21 was supported by a male patient, "BB", with XL-RP, DMD, CGD, McLeod phenotype and a deletion of Xp21 (Francke et al. 1985).

1.3.2 Choroideremia

Clinical features

Choroideremia is a chorioretinal disorder which exhibits an X-chromosomal pattern of inheritance (Goedbloed 1942; Waardenburg 1942; McCulloch and McCulloch 1948). The disease was first described by Mauthner (1872) who named the disease because of presumed congenital absence of the choroid. Later on, several investigators showed that the choroid is present in affected young males, and that this disease in fact is characterised by progressive degeneration of the retinal pigment epithelium (RPE), choroid, and retina. They considered the RPE to be the primary site of the defect, and therefore renamed this disorder tapeto-choroidal dystrophy (TCD) (Waardenburg 1958; Pameijer 1960). According to Heckenlively (1988), TCD is the most frequent of all X-linked types of chorioretinal degeneration, with an estimated frequency of 1 in 70.000 to 1 in 100.000.

The complete clinical picture can only be seen in affected males. Carrier females show striking but much less pronounced fundus changes which, with a few exceptions, cause no obvious vision impairment. The clinical picture and rate of progression can vary between affected individuals from different families, but also within the same family. At an early stage, affected males show non-specific pigment mottling in the midperiphery. Gradually, there is atrophy of the RPE, which is accompanied or directly followed by atrophy of the capillaries of the choroid. These initial fundus changes cause a diffuse dysfunction of the photoreceptors, and as a result,

patients experience night blindness. The age of onset of symptoms ranges from 3 to 40 years, with a peak between 10 and 30 (Rubin et al. 1966). Central visual acuity is normal and remains unaffected until late in the disease. At a later stage, outer retinal layers and large choroidal vessels become atrophic, first in the midperiphery, and progressing centrally towards the macular region. Consequently, vision deteriorates and progresses to so called "tunnel vision". Retinal veins appear normal, even in advanced stages, but retinal arteries can be somewhat attenuated (Bedell 1937; Cameron et al. 1987). Eventually, the entire fundus shows the yellowish-white reflex of the sclera, and about 35 years after the onset of symptoms, there is complete loss of vision (Rubin et al. 1966).

In female carriers, the expression of the disease is very variable. In the majority of cases, a characteristic mottling in the midperiphery or posterior pole can be observed, which closely resembles the fundus anomalies observed in affected young males (Pameijer et al. 1960; Krill 1967; McCulloch 1969). The fundus signs in these carriers are more or less stationary, and visual function remains good. The patchy changes in the RPE of these females are thought to reflect random X-inactivation. In some female carriers, the visual field defect resembles that of middle-aged male TCD patients (Fraser and Friedman 1968; Harris and Miller 1968).

Pathogenesis

Fundus changes characteristic for TCD patients or carrier females have been observed in very early childhood (Kurstjens 1965; McCulloch and McCulloch 1948). These findings suggest, but do not prove, that pathological processes have already started at, or even before, birth. The site of the primary lesion has been a subject of speculation since the disease was first described by Mauthner (1872). Pathologic studies have been hampered by the low incidence of TCD. The majority of donor eyes that have been examined showed an advanced or end-stage of the disease thus providing no insight into the very early changes associated with TCD. Based on ophthalmoscopic

investigations in male patients and carrier females, the RPE appears to be the tissue that is affected first (Waardenburg 1942; Pameijer 1960; McCulloch 1969). The RPE already shows pigmentary changes at a stage when the choroid and retina are still normal. This finding was corroborated by fluorescence angiography studies, which can visualize RPE and choriocapillaris loss at a very early stage (Hammerstein et al. 1979; Hammerstein and Bohm 1985).

As the fundus changes in carrier females resemble those of affected young males, some investigators believe that these changes provide a better clue to the early pathological processes. Light- and electron-microscopical studies performed on postmortem eyes of a 68 y old female carrier by Ghosh et al. (1988), disclosed a patchy loss of the retinal outer cell layer. Apart from a generalized thinning, the RPE only showed areas of depigmentation. The patches of altered tissue in retina and RPE were comparable in size. However, because of artificial detachment of the retina from the RPE/choroid, no conclusions could be drawn with respect to their spatial relation. No atrophy of the capillaries of the choroid could be seen, which prompted the authors to suggest that the primary defect should be located at the retina-RPE interface. A comparable study performed by Flannery and coworkers (1989) did not show such a strict correlation between areas of depigmented RPE and photoreceptor loss in the retina. In contrast, they observed that choroidal capillaries lying adjacent to abnormal receptor populations were reduced in number or practically absent. Those that were present in these patches showed reduced luminal diameter and sparse fenestrae.
A location of the primary defect in the choroid has also been suggested by Cameron et al. (1987), who observed abnormalities throughout the entire uveal tract (choroid, iris, ciliary body) in a male TCD patient. Hypoproduction of the basement membrane of vascular endothelial cells was not only associated with loss of the RPE and retina in the posterior uveal tract, but also by loss of dilator muscle and flattening of the iris pigment epithelium in the anterior uveal tract. No age-matched

normal eyes were studied in parallel, which possibly could rule out uveal changes related to the age (66 y) of this individual. The inner retinal layers and RPE are nourished by the choroid, and hence, absence of the latter tissue would very likely result in the degeneration of the RPE and retina. Whether this holds also for the reverse situation in which the RPE or parts of the retina degenerate is more difficult to assess.

In conclusion, postmortem eye studies have not provided conclusive evidence with respect to the location of the initial lesion, nor have they resulted in the identification of the biochemical defect underlying TCD.

Localization of the TCD gene

Although choroideremia was first described by Mauthner in 1872, its X-linked mode of transmission was not established until 70 years later by Goedbloed (1942) and by Waardenburg (1942). Initial studies showed that TCD was not linked to the Xg blood group locus (Other et al. 1968; Bell and McCulloch 1971), which maps to the distal part of the short arm of the X-chromosome. More recent studies using DNA markers have localized TCD to Xq13-q22 near DXYS1 (Lewis et al. 1985; Nussbaum et al. 1985). Subsequent studies showed that the TCD locus is closely, but not absolutely, linked to PGK1, DXS72, DXS95, DXYS1, DXYS5, and DXYS12 (Jay et al. 1986; Schwartz et al. 1986; Lesko et al. 1987; Sankila et al. 1987,1989; Wright et al. 1990). The combined lod score between DXYS1 and TCD is 32.3 at a distance of 3 cM (Davies et al. 1987). Due to relatively low recombination frequencies in the Xq13-q21 region (Drayna and White 1985) it was very difficult to establish the position of the TCD locus with respect to the above mentioned markers. In a family that was informative for DXYS1, MacDonald and coworkers (1987) found 2 cross-overs in 9 phase-known meioses. Both DXYS1 and DXS3 showed recombination with the disease locus. Given the small genetic distance between both markers (0-5 cM, Drayna and White 1985; Lesko et al. 1987), this strongly argued against a location of the TCD

gene between DXYS1 and DXS3. Placing TCD proximal to DXYS1 is, however, in apparent conflict with the order proposed by Gal et al. (1986a), who positioned the TCD locus distal to DXS3. Their gene order was based on haplotype analyses in two small families. On the basis of genotype information given for individuals of the pedigrees, a location of the TCD locus proximal to DXS3 is as likely as a position distal to this marker. Although genetic heterogeneity for TCD can not be strictly excluded, most data can be reconciled with only one TCD gene.

An alternative approach for fine mapping of the TCD gene emerged with the identification of deletions in the Xq21 region. The first deletion spanning part of Xq21 was described in 1983 by Tabor and colleagues in a male (NP), who suffered from cleft lip and palate, agenesis of the corpus callosum, and severe mental retardation. After the discovery of linkage between DXYS1 and the TCD locus (Nussbaum et al. 1985), NP and his family were reexamined ophthalmoscopically. His mother and sister were diagnosed as being carriers of TCD, while NP showed the typical fundus picture of a young male with TCD. Moreover, the deletion in his X-chromosome was shown to span DXYS1, corroborating the above mentioned linkage studies (Rosenberg et al. 1986, 1987; Schwartz et al. 1986). Three other microscopically visible deletions were found to be associated with TCD, mental retardation (MR), and in two cases (DM and XL62) with congenital deafness. Molecular analyses revealed that all microscopically visible deletions spanned DXYS1 and several other markers from Xq21 (Hodgson et al. 1987; Nussbaum et al. 1987; Schwartz et al 1988; Merry et al. 1989).

By employing the so called 'phenol enhanced reassociation technique' (PERT), Nussbaum and coworkers (1987) were able to generate a library of cloned DNA enriched for sequences that were deleted in a patient with TCD, congenital deafness and MR (patient XL45; Ayazi 1981). Two markers were identified that were deleted in patient XL45 and in patient XL62 with a somewhat larger Xq21 deletion. The deletion in XL45 did not

span DXYS1, indicating that the TCD locus is not located in the direct vicinity of DXYS1.

Recently, two balanced translocations have been found in females showing mild choroideremia. In both translocations, the breakpoint on the X-chromosome were situated in band q21.2, while the autosomal breakpoints were at 7p14 and 13p12, respectively (Kaplan et al. 1989; Siu et al. 1990). Apart from TCD, both females showed primary amenorrhea. The X-chromosomal breakpoint of the X/13 translocation could be positioned distal to the DXS165 locus, and proximal to the DXYS1 locus (Merry et al. 1990).

1.4 Outline of this thesis

The initial aim of this study was the systematic search for microdeletions in patients with complex X-linked disorders employing more than 200 DNA probes. In the course of these investigations it became apparent that cytogenetically visible, male viable deletions were clustered in the two largest Giemsa-dark staining regions of the X, Xp21 and Xq21 (chapter 2). Closer inspection revealed that many of the deletions spanning parts of the Xq21 band were associated with choroideremia and less frequently with congenital deafness and mental retardation (chapter 3). This finding prompted us to concentrate our studies on the molecular characterization of the Xq21 region and in particular, on the segment carrying the choroideremia gene. Therefore, the major part of this thesis deals with the fine mapping of the TCD gene region, and with our attempts to isolate this locus by means of positional cloning strategies (chapters 4 to 8). These studies have culminated in the isolation and characterization of cDNA clones from a gene that overlaps this region and is a likely candidate for TCD (chapter 9).

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Chapter 2

Molecular analysis of male-viable deletions and duplications allows ordering of 52 DNA probes on proximal Xq

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Am. J. Hum. Genet. 43:452-461,1988

Molecular Analysis of Male-viable Deletions and Duplications Allows Ordering of 52 DNA Probes on Proximal Xq

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Summary

While performing a systematic search for chromosomal microdeletions in patients with clinically complex X-linked syndromes, we have observed that large male-viable deletions and duplications are clustered in heterochromatic regions of the X chromosome. Apart from the Xp21 band, where numerous deletions have been found that encompass the Duchenne muscular dystrophy gene, an increasing number of deletions and duplications have been observed that span (part of) the Xq21 segment. To refine the molecular and genetic map of this region, we have employed 52 cloned single-copy DNA sequences from the Xcen-q22 segment to characterize two partly overlapping tandem duplications and two interstitial deletions on the proximal long arm of the human X chromosome. Together with a panel of somatic cell hybrids that had been described earlier, these four rearrangements enabled us to order the 52 probes into nine different groups and to narrow the regional assignment of several genes, including those for tapetochoroidal dystrophy and anhidrotic ectodermal dysplasia.

Introduction

The practical value of X-linked DNA markers for diagnosis and molecular elucidation of X-linked gene defects is limited by two fundamental problems. First, accurate estimation of small genetic distances between a given pair of syntenic genes requires that their segregation pattern be studied in numerous large families. In the absence of recombination, several hundred doubly informative individuals have to be examined to establish that the true genetic distance does not exceed 1 centimorgan (cM). Given the rarity of most Mendelian disorders, this implies that reliable estimation of the risk of recombination between a disease locus and a closely linked diagnostic marker is not possible. Second, there is no universally applicable formula to convert genetic distances into physical distances, and, in general, even very small genetic distances are still too large to be bridged by conventional molecular cloning. For a variety of X-linked defects, a solution to these problems has come from the analysis of clinically complex, X-linked syndromes that could be explained as small X-chromosomal deletions encompassing several genes and random DNA probes (Francke 1984; Lange et al. 1985). Minute deletions have been employed very successfully to identify useful diagnostic markers in the vicinity of the Duchenne muscular dystrophy (DMD) gene and elsewhere on the X chromosome, and deletions have played a crucial role for the isolation, by reverse genetics strategies, of the genes for DMD (Monaco et al. 1985) and chronic granulomatous disease (Royer-Pokora et al. 1986).

In the course of family studies, our group encountered various microdeletions in patients with complex X-linked disorders (Gal et al. 1985, 1986; Wieringa et al. 1985*a*, 1985*b*). These findings encouraged us to perform a systematic search for minute deletions in clinically complex X-linked syndromes by employing X chromosome–specific DNA sequences as probes. Since

Received February 17, 1988, revision received May 5, 1988

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0002-9297/88/4304 0012$02 00

several of the deletions that had been described were detectable by cytogenetic examination (Francke 1984; Francke et al. 1985; Wieringa et al. 1985*a*, 1985*b*), we speculated that, on average, deletions spanning several genes might encompass at least 1 million bp. Assuming that the human X chromosome comprises 200 million bp, we reasoned that 200 random probes should suffice to detect more than 60% of all deletions spanning several X-chromosomal genes, and this convinced us that such a search would be economically and logistically feasible. Therefore, in collaboration with several European and American colleagues, we collected DNA, blood, or cell lines from patients with X-linked syndromes that could be interpreted either as combinations of several X-linked diseases or as known X-linked disorders with additional clinical features. In parallel, numerous X chromosome–specific DNA probes were isolated and obtained from various laboratories, characterized, and, if necessary, regionally assigned by employing a panel of somatic cell hybrids (Wieacker et al. 1984).

While this work was in progress, several other male-viable deletions were found (Tabor et al. 1983; Old et al 1985; Bartley et al. 1986; Hodgson et al. 1987; Nussbaum et al. 1987), and it became apparent that large deletions are not evenly distributed on the human X. Almost invariably, deletions that can be detected by cytogenetic analysis map within the two most prominent, dark-staining Giemsa bands of the human X chromosome, Xp21 or Xq21. Because dark Giemsa bands are thought to be relatively devoid of functional genes (Kurnit and Hoehn 1979), we speculated that in these regions male-viable deletions might be larger than average. It followed that the inverse might be true for the remainder of the X, suggesting that detection of deletions outside these regions would require many more probes than originally anticipated. To some extent, this speculation was corroborated by the limited success of our deletion screening, which revealed only a single additional deletion (P. Braakhekke, personal communication). Since significant further enlargement of the probe panel did not seem practicable, we felt that instead we should concentrate on specific segments of the X and on complex syndromes comprising clinical features of regionally assigned gene defects. In addition, we decided that, apart from deletion screening, we would employ the available probes for the molecular analysis of previously described structural rearrangements of the X chromosome. The present paper describes the molecular analysis of male-viable deletions and duplications spanning several gene loci on the proximal long arm of the X chromosome, as well as the fine-mapping of 52 cloned DNA sequences in the Xcen-q22 segment.

Material and Methods

Clinical and Cytogenetic Data

Patient C.N. (Cremers et al. 1987*b*) suffered from muscular hypotony, growth retardation, psychomotor retardation, cryptorchidism, and Pelizaeus-Merzbacher disease (PMD) Cytogenetic and molecular analyses revealed a duplication of the Xq21-Xq22 segment, resulting from unequal crossing-over between the two maternal X chromosomes.

Patient K.M. (Vejerslev et al. 1985) showed psychomotor retardation, cryptorchidism, and various minor congenital malformations. Cytogenetic analysis suggested an inherited tandem duplication of the Xq13.1-q21.2 segment, but an insertion of autosomal material into the proximal long arm of the X chromosome could not be excluded.

Patient N.P. (Tabor et al. 1983) had cleft lip and palate (CLP), agenesis of the corpus callosum, and mental retardation. At the age of 5, retinal changes indicative for choroideremia were observed (Rosenberg et al. 1986; Schwartz et al. 1986). Cytogenetic and molecular examination showed an Xq21.1-q21.33 deletion which spanned the DXYS1 locus and was also present in N.P.'s mother (Rosenberg et al. 1986).

Patient R.v.D. is a mentally retarded boy with congenital heart defect (VSD), facial and skull deformities, frontal lobe atrophy, and various minor anomalies. His hearing is severely impaired (up to 75 dB), but even at the age of 10 ophthalmologic examination yielded no clinical signs of choroideremia. Chromosome analysis revealed an interstitial deletion encompassing a large portion of the Xq21 band and possibly part of band Xq22 (fig. 1). The same deletion was found in his mother who, like R.v.D., has a normal ocular fundus (J. Beverstock, personal communication).

Southern Blot Analysis

Chromosomal DNA from peripheral blood, Epstein-Barr virus–immortalized B cells, or fibroblast cell lines was isolated according to the method of Aldridge et al. (1984), with minor modifications. Restriction endonuclease–cleaved chromosomal DNA was resolved electrophoretically on a 0.7% (w/v) agarose gel. Following depurination in 0.15 N HCl for 10 min, DNA was denatured in 0.4 N NaOH and blotted onto a ny-

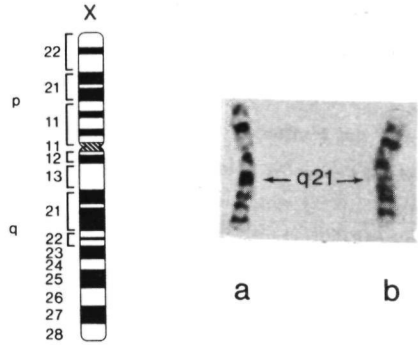

Figure 1 Cytogenetic analysis of the X chromosome of a normal male (*a*) and R.v.D. (*b*). GTG-banding reveals an interstitial deletion within Xq21, possibly extending into Xq22.

lon membrane (BioTrace,® Gelman Sciences, Inc.), using the same solution. Probe insert DNAs were isolated from low-gel-temperature agarose gels and labeled by primed synthesis with Klenow DNA polymerase I and α-^{32}P-dCTP, as described by Feinberg and Vogelstein (1983, 1984). Prehybridization of the DNA blots was done at 65 C for 6–18 h in 6 × SSC, 0.1% (w/v) BSA, 0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidone, 10% (w/v) dextran sulfate, 0.2% (w/v) SDS, and 250 μg sonicated, single-stranded herring sperm DNA/ml. Hybridization was performed for 16–20 h at 65 C in the same solution at a probe concentration of 1–3 ng/ml (2–5 × 10^8 cpm ^{32}P/μg). Washing was done at 65 C with stringencies increasing stepwise from 2 × SSC/0.5% (w/v) SDS to 0.1 × SSC/0.5% (w/v) SDS. Filters were rinsed in 2 × SSC and exposed to Kodak X-Omat film for 8 h–3 days at −80 C, using two intensifying screens.

Isolation of Single-Copy Sequences from Repetitive Probes

For some probes containing low or moderately repetitive X-chromosomal inserts, various insert segments were tested to obtain unambiguously interpretable hybridization patterns for signal density scanning. To this end, 2 μg of plasmid DNA was digested with endonuclease *Sau*3A and fragments were separated on a 1.5% (w/v) low-gel-temperature agarose gel. Distinct nonvector fragments were isolated, labeled with α-^{32}P-dCTP as described above, and hybridized to *Eco*RI-digested human control DNA. Unique sequences of probes pXG8b (0.6 kb), pXG12 (1.3 kb), p722 (1.2 kb), p776 (0.4 kb), and pTAK2 (1.4 kb) subsequently were used in the analysis of the rearrangements throughout this study. Occasionally, as for probes pX65H7, pX104f, pF1, and pF8, which failed to yield single-copy *Sau*3A subfragments, labeled insert DNAs were prehybridized to sheared human DNA (Cot = 3,600 mg × min/ml), essentially as described by Litt and White (1985), to reduce background hybridization.

Dosage Determination

Signal intensities were quantitated using an LKB 2202 Ultroscan Laser Densitometer (scan speed 20 mm/min, focus 50 μm). Hybridization signals obtained with probe p8 (DXS1), probe p708 (DXS82), or Y-specific signals (i.e., the 15-kbp *Taq*I band detected with pDP34 [DXYS1]) were used as internal references, and signal ratios were normalized through comparison with ratios of male control DNA. Values ranging from 0.7 to 1.3 and from 1.7 to 2.3 were considered to represent one or two copies, respectively, of the relevant DNA sequence.

Results

All 52 X-specific probes employed in this study, as well as their map positions, are listed in figure 2. For several of these, regional assignments have been reported elsewhere (Chance et al. 1983; Goodfellow et al. 1985; Willard and Riordan 1985; Riddell et al. 1986; Cremers et al. 1987*b*, 1987*c*). Others were regionally mapped by making use of a cytogenetically reanalyzed subset of the hybrid cell lines originally described by Wieacker et al. (1984). Recent reexamination of the breakpoint in hybrid 749, formerly given as Xq12 (Gerald and Brown 1974), has indicated that it may be at the interface of bands q12 and q13 or even at q13.1 (J. Zonana, personal communication). Therefore, the positioning

Figure 2. Signal dosage determination of hybridization intensities for probes located in the proximal Xq region. Horizontal lines indicate the cytogenetic assignment of the various breakpoints employed in this study. Lines with arrowheads at the right indicate the breakpoints in the hybrid cell lines, whereas lines without arrowheads indicate duplication and deletion breakpoints. The order of probes within each segment is arbitrary. The regional assignments of probes given in the third column refer to (a) the present study, (b) Cremers et al. (1987*b*), (c) Goodfellow et al. (1985), (d) Chance et al. (1983), (e) Willard et al. (1985), (f) Riddell et al. (1986), and (g) Willard and Riordan (1985). Probes cpX8, cpX12, cpX58, cpX205, cpX210, and cpX251 were isolated by one of us (M.H.H.); pXG8b was a gift from P. Szabo, New York; pF1 and pF8 were provided by T. A. Kruse, Aarhus. Quantitation of signal intensities was as described in Material and Methods.

HGM8 nomenclature	Probe designation	Regional assignment		Signal dosage duplications C.N.	K.M.	deletions N.P.	R.v.D.
DXS62	MGU22	p11-q12	(c)	+	+	+	+
	cpX8	p11.1-q12	(a)	+	+	+	+
	cpX210	p11.1-q12	(a)	+	+	+	+
DXS136	pX12g	cen-q12	(c)	+	+	+	+
DXS1	p8	q11-q12	(a,c)	+	+	+	+
DXS153	cX37.1	p11.1-q12	(a,c)	+	++	+	+
	cpX12	p11.1-q12	(a)	+	++	+	+
DXS132	cpX23	p11.1-q12	(a,c)	+	++	+	+
DXS133	cpX30	p11.1-q12	(a,c)	+	++	+	+
	cpX58	p11.1-q12	(a)	+	++	+	+
DXS106	cpX203	p11.1-q12	(b,c)	+	++	+	+
	cpX205	p11.1-q12	(a)	+	++	+	+
PGK1P1	pHPGK-7e	q11-q12	(d,e)	+	++	+	+
	cpX251	p11.1-q12	(a)	+	++	+	+
DXS162	cpX289	p11.1-q12	(a)	+	++	+	+
DXS171	pX63c	q12-q13	(a,c)	+	++	+	+
DXS131	cpX21	q12-q21.3	(b,c)	+	++	+	+
PGK1	pHPGK-7e	q12-q13	(d,e)	+	++	+	+
DXS159	cpX73	q12-q21.3	(b,c)	+	++	+	+
DXS135	cpX93	q12-q21.3	(a,c)	+	++	+	+
DXS56	pL2.98	q12-q21.3	(c)	+	++	+	+
DXS128	cX65.3	q12-q21.3	(b,c)	+	++	+	+
DXS227	p1bB4	q12-q22	(a)	+	++	+	+
DXS169	pX104f	q13-q21.1	(f)	++	++	-	-
DXS72	pX65H7	q13-q22	(c)	++	++	-	-
DXYS1	pDP34	q13-q21	(c)	++	+	-	-
DXYS5	p47b	q12-q22	(c)	++	+	-	-
DXYS12	pSt25.2	q13-q22	(c)	++	+	-	-
DXYS13	pTAK2	q12-q22	(b)	++	+	-	-
DXS165	p1bD5	q12-q21.3	(c)	++	+	-	-
DXS95	pXG7c	q21	(a,c)	++	+	-	-
	pXG8b	q12-q22	(a)	++	+	-	-
DXS110	p722	p11.1-q21.3	(c)	++	+	-	-
DXS121	p784	q12-q21.3	(b,c)	++	+	-	-
DXS214	pPA20	q12-q22	(a)	++	+	-	-
DXS73	pX20R42	q13-q22	(c)	++	+	-	-
DXS3	p19.2	q21.3-q22	(c)	++	+	-	-
DXS112	p753	q21.3-q22	(b,c)	++	+	-	-
DXS96	pXG3b	q12-q22	(b)	++	+	+	-
DXS118	p776	q21.3-q22	(b,c)	++	+	+	-
	pF1	q12-q22	(a)	++	+	+	-
	pF8	q12-q22	(a)	++	+	+	-
DXS94	pXG12	q12-q22	(b)	++	+	+	+
DXS17	pS9	q21.3-q22	(c)	++	+	+	+
DXS147	cX4.4	q21.3-q22	(c)	++	+	+	+
PLP	pLP1	q13-q22	(g)	++	+	+	+
DXS101	cX52.5	q21.3-q22	(c)	++	+	+	+
DXS211	pD3	q13-q22	(b)	++	+	+	+
DXS24	pX-5	q13-q22	(c)	++	+	+	+
DXS173	pX76a	q22	(b,f)	++	+	+	+
DXS111	p733	q22-q24	(c)	++	+	+	+
DXS82	p708	q22-q24	(c)	+	+	+	+
DXS11	p22.33	q24-q26	(c)	+	+	+	+

of this breakpoint in figure 2 may be somewhat arbitrary, and the same applies to the hybrid 676 × 175K37 breakpoint, whose exact location within band Xq22 is not known.

Molecular analysis of the Xq-proximal rearrangements enabled us to define a total of six new intervals spanning this chromosome area. Results from hybridization with representative probes from within each of these intervals are shown in figures 3*a* and 3*b*. Figure 2 summarizes the quantitation data obtained from densitometric scanning analysis. Out of the 52 sequences tested, 19 were duplicated in K.M.'s DNA. Therefore, in accordance with the cytogenetic findings of Vejerslev et al. (1985), these probes were tentatively assigned to the Xq13.1-q21.2 segment. In C.N., 28 probe sequences could be assigned to the duplicated Xq21.1-q22.3 segment. Two of these, pX65H7 (DXS72) and pX104f (DXS169), were also duplicated in K.M., which indicated that there is a small but detectable overlap between these duplications. As is shown in figure 3*b*, an extremely complex array of bands of single and double intensities was obtained with probe pHPGK-7e (PGK1). Of these, the 1.0- and 5.0-kbp signals, which originate from chromosome 19 and chromosome 6, respectively (Gartler et al. 1985), were used as reference signals for quantitation of X-signal dosage. The X-specific signal at 7.6 kbp is partly obscured by a signal of identical size originating from chromosome 6 (Michelson et al. 1985). Furthermore, the X-specific signals at 0.6, 2.7, and 5.5 kbp represent two loci at proximal Xq, namely, the PGK1 gene at q12-q13 and a PGK pseudogene (PGK1P1), tentatively assigned to q11–q12 (Chance et al. 1983; Willard et al. 1985). As can be inferred from the autoradiograph, clearly all X-specific PGK signals were found to be of double intensity in K.M.'s DNA but not in C.N.'s DNA. We have confirmed this result by a similar analysis using restriction enzyme *Eco*RI to distinguish between the authentic and the pseudo-PGK signals from Xq (not shown).

In a previous study (Cremers et al. 1987*b*), we had concluded that the two X/Y homologous sequences, DXYS1 and DXYS5, one of which had been assigned previously to the interface of bands Xq13 and q21 (Page et al. 1984), map proximal to the duplicated DNA segment of patient C.N. Still, neither of the two was found to be located on the duplicated segment of patient K.M. Critical reexamination of our previous results indicated that, contrary to that previous report, all four X-Y homologous loci tested (i.e., DXYS1, DXYS5, DXYS12, and DXYS13) map inside the C.N. duplication and thus more distally than had been indicated previously.

This is corroborated by the failure of all four probes to yield X chromosome–specific hybridization signals in the DNA of the two deletion patients, N.P. and R.v.D. (figs. 2, 3*a*). In N.P., cytogenetic analysis had revealed a large deletion spanning the q21.1-q21.33 segment (Tabor et al. 1983; Rosenberg et al. 1986), and, in R.v.D., there was cytogenetic evidence for a deletion encompassing part of Xq21 and q22 (see Material and Methods; J. Beverstock, personal communication). Our hybridization experiments suggest that the R.v.D. deletion is slightly larger than the N.P. deletion. The former spans 19 and the latter 15 DNA loci, all of which are located on the segment that is duplicated in patient C.N. Both deletions overlap the K.M. duplication, as evidenced by the fact that they encompass two markers, pX65H7 (DXS72) and pX104f (DXS169), which are duplicated in the DNA of patient K.M. In contrast, none of the four markers that are deleted in R.v.D. but not in N.P.—i.e., pXG3b (DXS96), p776 (DXS118), pF1, and pF8—are duplicated in this patient. This indicates that all of these four markers are situated at the distal end of the R.v.D. deletion. As yet we have not identified probes that distinguish between the proximal endpoints of the two deletions and the C.N. duplication in the vicinity of the Xq13.3-q21.1 boundary.

Previous studies had indicated that, probably as a result of unequal crossing-over between the two maternal X chromosomes, C.N. is heterozygous for several polymorphic DNA markers from the proximal Xq (Cremers et al. 1987*b*). In K.M., the search for heterozygosity was unsuccessful with four probes—cpX203 (DXS106), cpx289 (DXS162), cpX93 (DX135), and pHPGK-7e (PGK1)—all located on the duplicated segment and known to detect two-allelic RFLPs. Given the frequencies of the rare alleles—frequencies that are .35, .33, .06, and .40, respectively—the probability of two different X chromosomes carrying identical alleles at all four loci tested is about 14%. Therefore, our findings suggest that the K.M. duplication did not arise by unequal crossing-over but by a different mechanism.

Discussion

Owing to their dual function as genetic and physical signposts, DNA markers play an essential role in the construction and alignment of genetic and physical chromosome maps. While the goal of covering all human chromosomes with a network of closely linked DNA markers has almost been reached (Donis-Keller et al. 1987; Nakamura et al. 1987), the accuracy of the physical map is still hampered by the limits of

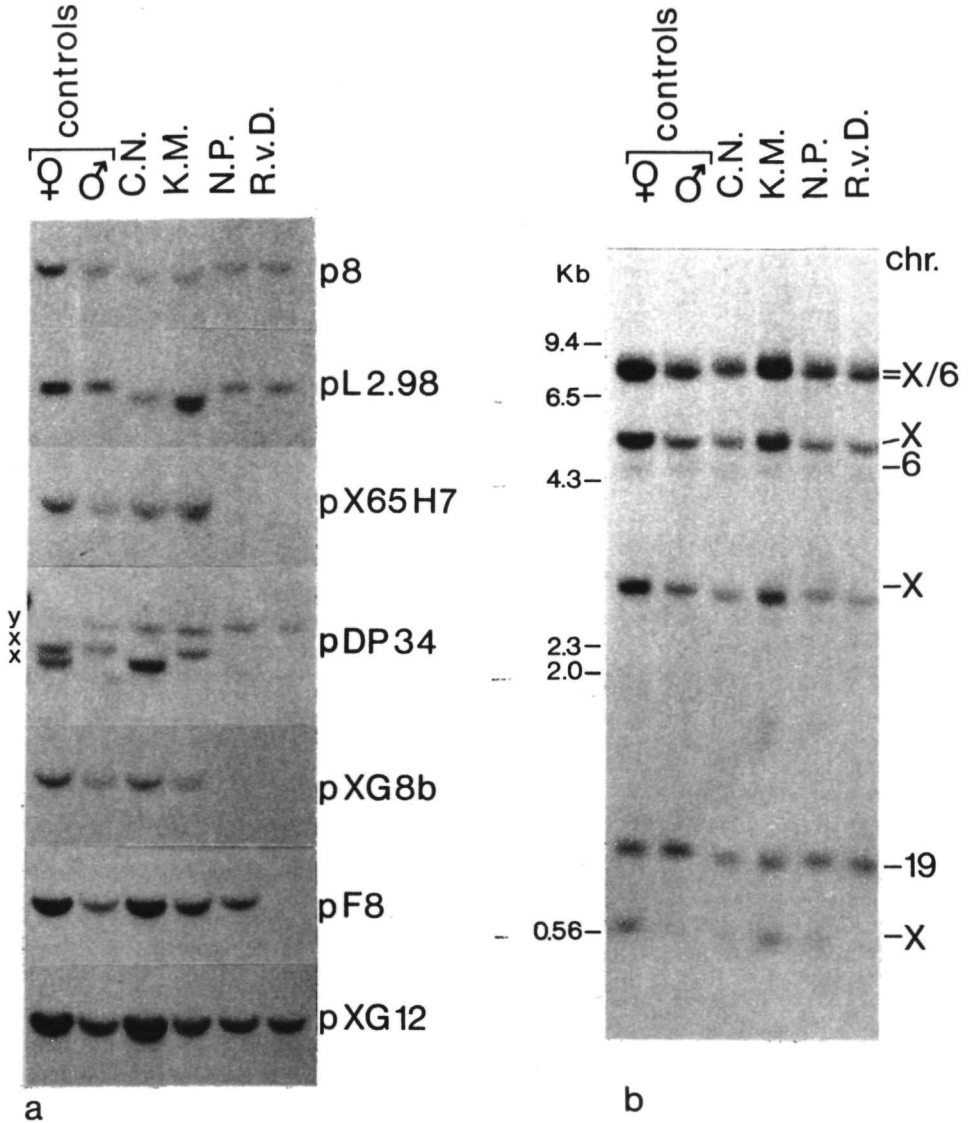

Figure 3 Southern blot analysis of genomic DNAs from patients with rearrangements at proximal Xq, using probes from within several intervals in the Xp11-Xq24 area. *a*, For all probes hybridization analysis was on *Eco*RI-digested (except for pDP34, which was digested with *Taq*I) genomic DNA of two controls (male and female) and four patients. Relevant sections from the various autoradiographs are arranged—according to the position of the probe—from proximal to distal (compare with fig. 2). *b*, Southern analysis using probe pHPGK-7e (PGK1). DNAs of patients and controls were digested with restriction enzyme *Hin*dIII. The chromosomal localisation of various X- and autosome-specific signals is indicated to the right. Size markers are given to the left.

cytogenetic resolution. Here we report on our attempt to correlate the size of two deletions and two duplications on the proximal long arm of the X chromosome with the presence or absence of specific single-copy DNA sequences. This has enabled us to define the endpoints of the duplicated or deleted segments and to refine the assignment of numerous DNA probes that had been mapped previously by employing a panel of somatic cell hybrids (Wieacker et al. 1984).

Probes in the Xcen–Xq22 region could be ordered into nine segments defined by eight discernible chromosome breakpoints (see fig. 2). This is two less than expected because the proximal borders of the deletions and of one duplication appear to coincide: all three are located between the DXS227 and DXS169 loci. Apart from coincidence, which seems highly implausible, or enhanced proneness of this region to chromosome breakage, for which there is no evidence, it is possible that DNA sequences from the chromosome segment that harbors these breakpoints may be underrepresented in our panel of probes. Alternatively, it is conceivable that there are functional constraints that prohibit a small region near the border of band Xq13 and Xq21 from being deleted or duplicated (although, for duplications, these constraints cannot be absolute because there is a small but detectable overlap between the two duplications: both encompass DXS72 and DXS169)

Though there is no evidence for obvious clustering or scarcity of probes within the Xcen-q22 segment, this possibility cannot be ruled out, because several of the breakpoints given may not be precisely defined owing to the limited resolution of conventional cytogenetic analysis Indeed, the recent observation (J. Zonana, personal communication) of a more distal localization of the breakpoint in hybrid 749 is supported by the fact that nine probes that map proximal to this breakpoint are still comprised in the duplicated Xq13.1-Xq21.2 segment of patient K.M. (Vejerslev et al. 1985, see fig. 2). Whether these nine probes are derived from nine different loci is, however, questionable, as all probes except cX37.1 were subcloned from pooled cosmids (Hofker et al. 1987). Nonetheless, based on the molecular analysis, our data suggest a more proximal location of the duplicated DNA segment in patient K.M. (Xq12.2-q21.1; see fig. 2). It is interesting that the 749 breakpoint may be located within the locus for AED since this hybrid had been originally established from fibroblasts of a female with an X-autosome translocation, t(X;9) (q12;p24) and AED (Grzeschik and Siniscalco 1976; K H. Grzeschik, personal communication). This would imply that the anhidrotic ectodermal dysplasia locus is flanked by the phosphoglycerate kinase (PGK1) gene and the PGK pseudogene (PGK1P1), which map distal and proximal to this breakpoint, respectively (Chance et al. 1983; see fig. 2).

Recently, Arveiler et al. (1987*b*) have presented a refined genetic map of the Xq11–q22 region which includes several of the probes dealt with in the present study. Our results are in keeping with their data, except for two loci. According to Arveiler et al., the DXS72 locus maps distal to the sequences within band Xq21 that show homology to the Y chromosome. In contrast, our study assigns this marker proximal to these X–Y homologous sequences, and this assignment is corroborated by linkage studies which point to a location proximal to the DXYS1 marker (B. van Oost, personal communication). Second, there is an inconsistency pertaining to the regional assignment of the DXS159 locus (probe cpX73). Using the same somatic cell hybrid line (749 or ANLY1), Arveiler et al place this marker proximal to the breakpoint while we have mapped it to the distal side. This discrepancy can only be resolved by assuming that Arveiler et al. have employed a different probe. In fact, this probe sequence detects a *Pst*I polymorphism (Arveiler et al. 1987*a*), whereas DXS159 is not polymorphic for this enzyme (M H.H , unpublished data).

Because of the strikingly different phenotypes associated with the two deletions studied, N.P. and R.v.D., it is tempting to speculate that they encompass several different genes. In part, this is corroborated by the cytogenetic finding that the R.v.D. deletion extends into the q22 band while the N P. deletion is confined to q21 (Tabor et al. 1983; Rosenberg et al. 1986). Our hybridization studies confirm these findings by defining four sequences—pXG3b (DXS96), p776 (DXS118), pF1, and pF8—that are only absent in the larger of the two deletions. These findings may account for the presence of deafness in patient R.v.D. and its apparent absence in patient N.P. Deafness due to stapes fixation is a well-known X-linked disorder (McKusick 30440; Cremers et al. 1985), and the gene responsible for this disorder has recently been assigned to the proximal long arm of the X chromosome by demonstration of linkage to the DXYS1 marker (H. G. Brunner, personal communication). Deafness with stapes fixation has also been described in another patient with a deletion encompassing part of the Xq21 band (Ayazi 1981; Nussbaum et al. 1987).

Presence of CLP, agenesis of the corpus callosum, and choroideremia in patient N.P.—and the absence of these features in R.v.D., the patient with the larger

deletion—points to the possibility that the N.P. deletion may extend farther proximal than the R.v.D. deletion, a possibility for which cytogenetic analysis provides some support. This would further substantiate our speculation that there is a gap in our probe panel and may assign the genes responsible for these differences to the segment between these two breakpoints. In particular, this may apply to a gene involved in the etiology of CLP, since linkage studies have recently mapped a cleft palate gene to the same chromosome region (Moore et al. 1987). On the other hand, deletions of the X-linked CLP gene may not always give rise to CLP. Indeed, CLP has not been found in any of the other large Xq21 deletions that have been described (Hodgson et al. 1987; Nussbaum et al. 1987). In contrast, tapetochoroidal dystrophy (TCD) has been observed repeatedly and is an almost constant feature of these deletions. Comparison of the relevant chromosomal breakpoints has provided clear evidence that TCD cannot be located near the interface of Xq13 and Xq21 but should map farther distal, probably within the Xq21.1-q21.2 segment (F.P.M.C., unpublished data). Therefore, it is likely that the TCD gene is deleted in R.v.D., too, and that the absence of clinical signs in this patient may be a consequence of his young age.

These examples illustrate the mutual usefulness of (1) DNA probes for the genetic and molecular characterization of chromosomal rearrangements and (2) duplications and deletions for the fine-mapping of probes. The precise regional assignment and ordering of 52 cloned DNA sequences from the proximal long arm of the X chromosome into nine different groups should provide the framework for the construction of a contiguous molecular map of this chromosome segment by field-inversion gel electrophoresis (FIGE; Carle et al. 1986) or related techniques. Moreover, the alignment achieved between the physical and the genetic map should facilitate the precise regional assignment of other genes and gene defects in this region. Known physical distances and gene orders will permit a more directed search for highly informative diagnostic markers, which appear to be less abundant on the X than on other human chromosomes (Hofker et al. 1986); and this information should be a great asset for the isolation of genes by chromosome walking and hopping strategies. For TCD, we have already been able to confirm this prediction by defining a DNA sequence in the immediate vicinity of the choroideremia gene, as evidenced by the fact that this sequence is deleted in two of eight TCD patients tested (Cremers et al. 1987*a*). Future research in our laboratory will concentrate (1) on the physical mapping of the TCD gene region by employing FIGE and related techniques and, eventually, (2) on the isolation of the TCD gene itself.

Acknowledgments

The authors are indebted to Drs I Balazs, G. A. Bruns, H. J. Cooke, K. E. Davies, T. A. Kruse, S. A. Latt, J. L. Mandel, A. M Michelson, D. C. Page, J. R Riordan, K. Smith, P. Szabo, J. Weissenbach, and B. N. White for supplying DNA probes. We thank F. Oerlemans for technical assistance in cell culturing and Dr. D. F. C. M. Smeets for cytogenetic analysis. This work was supported by grant 900–504–064 from the Dutch Stichting voor Medisch Onderzoek en Gezondheidszorg (Medigon-ZWO) and by Deutsche Forschungsgemeinschaft (DFG) grant Ro389/13-3.

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Chapter 3

Physical fine mapping of the choroideremia locus using Xq21 deletions associated with complex syndromes

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Genomics 4:41-46,1989

Physical Fine Mapping of the Choroideremia Locus Using Xq21 Deletions Associated with Complex Syndromes

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Received July 11, 1988, revised September 1, 1988

Characterization of several male-viable deletions and duplications with 20 random DNA probes has enabled us to subdivide the Xq21 region into seven discernible intervals. Almost all of the deletions spanning part of Xq21 are associated with choroideremia and mental retardation, with deafness being another common feature. The gene locus for choroideremia was assigned to interval 3 spanning the loci *DXS95*, *DXS165*, and *DXS233*. Genes for X-linked deafness and mental retardation were tentatively assigned to interval 2. Deletions of intervals 4 through 7 were not associated with any clinical abnormality. We have constructed a preliminary long-range restriction map of intervals 2 and 3 using field-inversion gel electrophoresis. The *DXS232*, *DXS121*, and *DXS233* loci are located on the same *Sfi*I fragment, whereas the *DXS165* and *DXS95* loci could not be linked to this cluster using *Sfi*I and *Sal*I.

INTRODUCTION

Choroideremia is an X-linked retinal dystrophy in which affected males suffer from progressive night blindness and visual field constriction, leading to blindness by the third to fourth decade of life (Goedbloed, 1942; Waardenburg, 1942; McCulloch and McCulloch, 1948; Sorsby *et al.*, 1952). Female carriers, although normally asymptomatic, can be detected by characteristic patchy changes in the retinal pigmented epithelium that reflect random X inactivation.

The gene locus for choroideremia (tapetochoroidal dystrophy: TCD) has been assigned to the proximal long arm of the X chromosome through demonstration of tight linkage with various restriction fragment length polymorphisms (RFLPs) all of which are located in the Xq13–q21 region (Nussbaum *et al.*, 1985; Schwartz *et al.*, 1986; Sankila *et al.*, 1987; Lesko *et al.*, 1987). More accurate mapping of the TCD gene has been hampered by relatively low recombination frequencies which are characteristic of this part of the X chromosome (Drayna and White, 1985; Lesko *et al.*, 1987; Arveiller *et al.*, 1987).

Recently, a solution to this problem has come from the analysis of a number of male-viable deletions spanning part of the proximal Xq region that were found to be associated with TCD and other features. Clinical, cytogenetic, and molecular characterizations of these deletions have allowed the isolation (Nussbaum *et al.*, 1987) and physical mapping of markers located in the close vicinity of the TCD locus (Schwartz *et al*, 1986, 1988; Hodgson *et al*, 1987; Cremers *et al.*, 1988). So far, however, the number of probes employed in each of these studies was too small compared with the size of the deletions to permit accurate physical mapping of the TCD gene itself. Moreover, none of the previous analyses dealt with more than two TCD deletions, which ruled out direct comparison and the definition of the smallest region of overlap.

Recently, we have ordered a total of 52 polymorphic and nonpolymorphic DNA probes on proximal Xq by making use of somatic cell hybrids and large interstitial duplications and deletions. These studies enabled us to assign the TCD gene, as well as 19 of these probes, to the Xq21 band (Cremers *et al.*, 1987a, 1988). In the present study we have employed all available Xq21 markers to characterize four additional TCD deletions that have been described previously (Hodgson *et al.*, 1987; Nussbaum *et al.*, 1987; Schwartz *et al.*, 1988). In this way, these probes could be assigned to several different intervals of the Xq21 band. Moreover, these analyses permitted accurate and unambiguous assignment of the TCD gene. In addition, small chromosomal regions that seem to play a role in the etiology of X-linked deafness and X-linked mental retardation could be defined. Finally, the large number of available probes

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enabled us to construct a preliminary large-scale restriction map surrounding the TCD locus using field-inversion gel electrophoresis (FIGE).

MATERIALS AND METHODS

Cytogenetic and Clinical Data

Table 1 summarizes the cytogenetic findings in the patients who were investigated in this study. Patient CN suffered from muscular hypotony, growth retardation, psychomotor retardation, cryptorchidism, and Pelizaeus–Merzbacher disease (Cremers *et al.*, 1987a). Patient KM showed psychomotor retardation, cryptorchidism, and various minor congenital malformations (Vejerslev *et al.*, 1985; Cremers *et al.*, 1988). Patient RvD is a mentally retarded boy with congenital heart defect (VSD), facial and skull deformities, frontal lobe atrophy, severe hearing loss (up to 75 dB), and various minor anomalies (Cremers *et al.*, 1988). Patient NP showed TCD, cleft lip and palate (CLP), agenesis of the corpus callosum, and severe mental retardation (Tabor *et al.*, 1983; Rosenberg *et al.*, 1986; Schwartz *et al.*, 1986, 1988). Patient MBU is a mildly retarded boy suffering from TCD (Hodgson *et al.*, 1987). Neither patient MBU nor patient NP is deaf. The unrelated patients XL62 and XL45 suffer from TCD, mental retardation, and congenital sensorineural deafness (Ayazi, 1981). Patient DM was diagnosed as having TCD, congenital sensorineural deafness, and mental retardation (Schwartz *et al.*, 1988).

DNA Markers

All probes employed in this study are listed in Fig. 1. The regional assignments based on structural X-chromosomal abnormalities (translocations, duplications, deletions) have been published elsewhere (Goodfellow *et al.*, 1985; Nussbaum *et al.*, 1987; Cremers *et al.*, 1987a, 1988). Probe pXG8b was kindly provided by P. Szabo, New York; pF1 and pF8 were gifts from T. A. Kruse, Aarhus.

TABLE 1

Summary of Cytogenetic Data

Patient	Rearrangement	Cytogenetic assignment	Refs.
CN	Duplication	q21.1–q22.3	(8, 10)
KM	Duplication	q12–q21.1	(10, 42)
MBU	Deletion	q21[a]	(20)
NP	Deletion	q21.1–q21.33	(10, 32, 39)
RvD	Deletion	q21.1–q22.1	(10)
XL62	Deletion	q21[a]	(30)
DM	Deletion	q21.2–q21.31	(37)
XL45	Deletion	q21[b]	(30)

[a] Accurate locations of deletion breakpoints were not established.
[b] Submicroscopic deletion (30).

Southern Blot Analysis

Chromosomal DNA was isolated according to Aldridge *et al.* (1984), with minor modifications. DNA (10 μg) was digested with the appropriate restriction enzyme(s), and fragments were resolved by electrophoresis and blotted on to Biotrace (Gelman Sciences Inc.) or GeneScreen Plus (NEN) membranes as described previously (Cremers *et al.*, 1988). Radioactive probes were prepared by *random oligonucleotide priming* (Feinberg and Vogelstein, 1983, 1984). Insert DNA was electrophoretically separated from plasmid vector and isolated from low-gelling-temperature agarose. Methods for (pre)hybridization and washing of filters have been published previously (Cremers *et al.*, 1988).

FIGE Analysis

Preparation of high-molecular-weight DNA in agarose blocks, restriction enzyme digestion, and FIGE gel electrophoresis were performed as described by Van Ommen and Verkerk (1986) and Carle *et al.* (1986). In brief, cells of the human cell line LCL127 containing four X chromosomes were embedded at a concentration of 7.5×10^6 cells/ml in 0.6% low-gelling-temperature agarose (0.3×10^6 cells/block). Blocks were incubated in 0.5 *M* EDTA (pH 8.0)/1% (w/v) sodium lauroyl sarcosine, 0.5 mg/ml proteinase K at 50°C for 48 h; washed several times with distilled water, twice with 10 m*M* Tris–HCl/0.5 m*M* EDTA (pH 8.0) (TE) plus 0.1 m*M* PMSF for 2 h, twice with TE for 2 h; and stored in 0.5 *M* EDTA at 4°C. Digestions were performed with 30–45 U of enzyme for 5 h in a volume of 100 μl. Blocks were washed in TE and transferred to preformed slots of a 1% (w/v) agarose gel. Gels were run in 0.5× TBE buffer (Van Ommen and Verkerk, 1986) for 22 h at 6°C and 160 V. Switching intervals increased linearly from 6 to 60 s for the forward phase and from 2 to 20 s for the reverse phase.

After staining with ethidium bromide, gels were photographed and prepared for DNA transfer by sequential incubation in 0.15 *M* HCl for 2× 10 min, 0.5 *N* NaOH/1.5 *M* NaCl for 2× 30 min, and 0.5 *M* Tris–HCl, pH 7.5/1.5 *M* NaCl for 2× 30 min. Transfer to Hybond-N membranes (Amersham) was performed in 10× SSC overnight. Binding of DNA and rehybridization were performed as recommended by the manufacturer.

RESULTS

Molecular Analysis of X-Linked Deletions and Duplications

Twenty DNA probes, all of which derived from the Xq21 segment, were employed to localize the break-

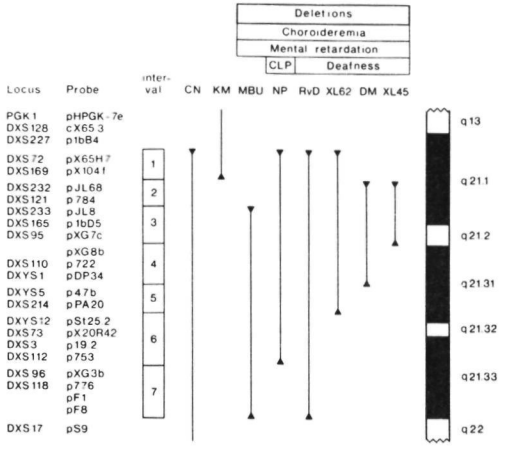

FIG. 1. Duplication and deletion map of the Xq21 band. Duplicated and deleted segments are indicated by vertical lines and breakpoints by arrowheads. The ordering of probes within each interval is arbitrary, except for the probes pJL68, p784, and pJL8, which are positioned in accordance with the results of FIGE analysis (Fig. 3).

points and to compare the sizes of two tandem duplications and six interstitial deletions spanning parts of the Xq21 band. In patients CN and KM, the copy numbers of the corresponding genomic sequences were determined by Southern blot hybridization. Signal dosage was quantitated by laser densitometry of the autoradiographs, as described previously (Cremers *et al.*, 1987a). CN and KM have large, partly overlapping duplications which share a small segment of Xq21.1, including the *DXS72* and *DXS169* loci, and extend into Xq22.3 and Xq12, respectively (Cremers *et al.*, 1987a, 1988; see also Fig. 1). The DNA of the six deletion patients was tested for the presence or the absence of hybridization signals. In this way, the available probes allowed us to subdivide the Xq21 band into seven separate intervals, as illustrated in Fig. 1. It is noteworthy that several of the breakpoints appear to coincide. We cannot distinguish between the proximal borders of three deletions (RvD, NP, and XL62) and the CN duplication. The same is true for the distal demarcation of the KM duplication and the proximal border of two other deletions (XL45 and DM) and for the distal breakpoints of the RvD and MBU deletions, respectively. All probes previously shown to be outside the RvD deletion (Cremers *et al.*, 1988) are also located outside the other deletions, as illustrated in Fig. 1 for *PGK1, DXS128, DXS227,* and *DXS17.*

Both X-chromosomal (p47b) and Y-chromosomal (p31) sequences of the *DXYS5* locus have been cloned (Goodfellow *et al.*, 1985). In this study, probe p47b is assigned to interval 5, distal to the DM deletion (Fig. 1). Recently, Schwartz *et al.* (1988) showed that probe p31 overlaps one of the deletion breakpoints in patient DM. Our data indicate that *DXYS5* spans the most distal of the two DM breakpoints. Probe 47b does not detect this breakpoint in *Taq*I-digested DNA and therefore it must be located distal to the X homolog of probe p31.

As to the location of the genes involved in these deletions, comparison with the respective clinical findings yields (nearly) consistent results. With one exception (RvD, see below), all deletions spanning interval 3 defined by probes pJL8 (*DXS233*), p1bD5 (*DXS165*), and pXG7c (*DXS95*) are associated with choroideremia, which assigns the TCD gene to this region. Similarly, the absence of a gene or genes in interval 2 seems to evoke deafness, and genes in this region appear to play a role in the etiology of mental retardation, too.

Large-Scale Restriction Map of the TCD "Critical" Region

The DNA markers that were shown to be located in the smallest syndromic TCD deletion (XL45, see Fig. 1), probes p784, pJL68, pJL8, p1bD5, and pXG7c, were hybridized to FIGE blots containing *Sfi*I, *Sal*I, or *Sfi*I/*Sal*I double digests of DNA of cell line LCL127. As illustrated in Fig. 2, pJL68, p784, and pJL8 detect identical *Sfi*I fragments. Moreover, p784 and pJL8 hybridize to identical *Sal*I and *Sfi*I/*Sal*I fragments, which indicates that the maximal physical distance between

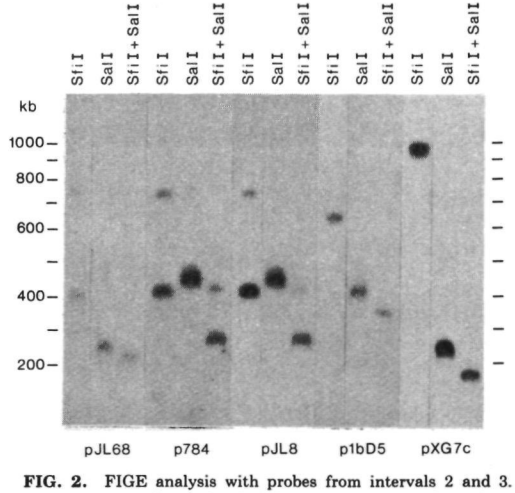

FIG. 2. FIGE analysis with probes from intervals 2 and 3. LCL127 DNA was digested with different enzymes as indicated for each lane. The scale shown on the left is based on the migration of the *Saccharomyces cerevisiae* chromosomes which were employed as size markers.

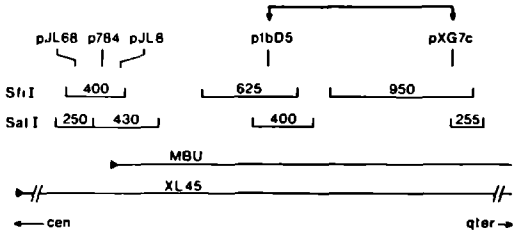

FIG. 3. Provisional large-scale restriction map of part of intervals 2 and 3 which span the TCD locus. *Sfi*I and *Sal*I fragment sizes are given in kb. The orientation and positioning of the fragments detected by P1bD5 and pXG7c are unknown.

these two markers should not exceed 220 kb. Probes p1bD5 and pXG7c detect clearly different *Sfi*I and *Sal*I fragments.

Recent studies by Julier and White (1988) have indicated that the mobility of restriction fragments in pulsed-field gel electrophoresis (PFGE) is affected by the amount of DNA applied to the gel. In our FIGE analysis *Sfi*I/*Sal*I fragments have a tendency to run more slowly; they comigrated with fragments that were on average 30–40 kb larger in size. This effect is not seen in *Sfi*I or *Sal*I single digests, probably because of a more even distribution of the DNA throughout the gel (unpublished results).

The FIGE data are summarized in Fig. 3. The orientation of the probe cluster pJL68–p784–pJL8 can be inferred from the fact that pJL8 is the only probe of that cluster mapping inside the MBU deletion (see Fig. 1; R. L. Nussbaum, unpublished results). In contrast, the orientation and positioning of the fragments detected by probes p1bD5 and pXG7c are not known with certainty because we have not yet been able to physically link these two probes with the nearest proximal marker, pJL8.

DISCUSSION

On the X chromosome, large male-viable deletions are clustered in the two most prominent dark-staining, and late-replicating, Giemsa (G) bands, Xp21 (Francke, 1984; Francke *et al.*, 1985; Old *et al.*, 1985; Bartley *et al.*, 1986) and Xq21 (Tabor *et al.*, 1983; Hodgson *et al.*, 1987; Nussbaum *et al.*, 1987; Schwartz *et al.*, 1988; Cremers *et al.*, 1988, this study). This is in keeping with previous studies which suggested, on the basis of karyotype–phenotype correlations, that Giemsa light-staining bands (reverse or R-bands) are enriched for expressed sequences, whereas G bands are genetically inert (Comings, 1974; Sanchez and Yunis, 1974; Yunis *et al.*, 1977; Korenberg *et al.*, 1978; Kurnit and Hoehn, 1979). A functional link between these observations may be provided by the accumulating evidence that (the initiation of) replication and transcription are partially dependent on the same DNA structures (e.g., see review by DePamphilis, 1988). For the Xq21 band, this correlation appears to hold, as evidenced by the clustering of large, male-viable deletions in this region and the conspicuous absence of (grave) clinical symptoms in these patients which is confirmed by the present study.

Characterization of several duplications and deletions with 20 probes derived from the Xq21 band has enabled us to subdivide this region into seven intervals and to determine their (centromere to telomere) orientation. Moreover, at least three disease loci could be assigned to two of these intervals through comparison with the associated clinical phenotypes. As shown in Fig. 1, the deletion in patient XL45, which spans intervals 2 and 3, is associated with TCD, deafness, and MR, whereas TCD is the only prominent clinical feature in patient MBU, whose deletion encompasses intervals 3 through 7. This suggests that the gene underlying TCD maps in interval 3, whereas intervals 4 through 7 are devoid of functionally important loci. TCD is a constant feature of all deletions examined that span interval 3, except for one patient, RvD. Here, the lack of conspicuous changes in the ocular fundi may be related to his youth (11 years). At this age, nonmanifestation of TCD in male carriers is not exceptional (Kärnä, 1986).

In general, deletions spanning interval 2 are associated with deafness. There is one patient (NP), however, who is not deaf despite a large deletion encompassing intervals 1 through 7. Thus it appears that deletion of interval 2 predisposes for, but does not necessarily cause, hearing impairment. The genetic classification of deafness is difficult because of its clinical variability. Congenital mixed, that is, conductive and sensorineural, deafness with stapes fixation (McKusick Catalog No. 30440) is the most frequent X-linked form of hearing impairment. Close linkage with the *PGK1* (Brunner *et al.*, 1988) and the *DXYS1* markers (P. Beighton, personal communication) has recently assigned this gene to the proximal Xq, which suggests that the same gene may be involved in the hearing impairment associated with Xq21 deletions. Indeed, stapes fixation has been established in patient XL45, but not in patients RvD, XL62, and DM in whom congenital sensorineural deafness has been reported. Therefore, the possibility that interval 2 carries more than one gene that may give rise to deafness cannot be excluded.

About 60% of all cases with X-linked MR are unrelated to the fragile Xq27 syndrome (Mikkelsen, 1987), and several forms of nonspecific X-linked MR have been reported. In addition, MR has been found in a large number of other complex X-linked syndromes (Tariverdian and Weck, 1982). MR is also a common feature in complex TCD deletions. A locus (or loci) for

MR can be assigned to interval 2 and/or interval 3, depending on the clinical findings in patient MBU, who is mildly retarded

Recently, linkage has been demonstrated between a gene defect causing X-linked cleft palate and ankyloglossia and the *DXYS1* marker on the proximal long arm of the X chromosome (Moore *et al*, 1987) In view of this finding, the observation that one of the deletion patients, NP, has (unilateral) cleft lip and palate may not be coincidental On the other hand, it is well established that X-linked forms of cleft lip and palate are very rare compared to multifactorial ones, and the functional role of this gene may be small given the absence of cleft lip and palate in all other patients with Xq21 deletions

Previously published cytogenetic data on the syndromic TCD deletions (Table 1) are corroborated by our physical mapping studies The MBU and XL62 deletions were estimated to span half of the Xq21 band (Hodgson *et al*, 1987, Nussbaum *et al*, 1987) On the basis of the relative map positions of the various breakpoints, we tentatively assign the MBU deletion to Xq21 2–21 33 and the XL62 deletion to Xq21 1–q21 31

As shown in Fig 1, deletion and duplication breakpoints in the Xq21 region appear to be distributed in a nonrandon fashion, as judged from the clustering of breakpoints which is most notable in the Xq21 1 band As shown by FIGE analysis, the *DXS121, DXS232,* and *DXS233* loci are located on the same 400-kb *Sfi*I fragment, whereas *DXS95* and *DXS165* are located on different *Sfi*I fragments This suggests that probe clustering is at least partly responsible for the observed clustering of breakpoints One of the DNA marker sequences employed in this study, *DXYS5*, is directly involved in chromosome breakage As shown by Schwartz *et al* (1988), locus *DXYS5* demarcates a deletion breakpoint in patient DM This finding is particularly notable in view of a previous report dealing with the participation of the *DXYS5* sequence in another chromosome rearrangement (Rouyer *et al*, 1987). This observation may indicate that some sequences are characterized by enhanced proneness to chromosome breakage

Physical linkage with TCD is probably closest for *DXS165,* as judged from our recent finding in patients with classical TCD of deletions spanning the *DXS165* locus but none of the other markers (Cremers *et al*, 1987b). As shown in Fig 1, this implies that all probes thought to be located in the vicinity of the TCD locus because of close linkage, namely, *DXYS1, DXYS12, DXS72,* and *PGK1* (Nussbaum *et al*, 1985, Schwartz *et al*, 1986, Lesko *et al*, 1987, Sankila *et al*, 1987), map outside the region that carries the TCD gene (interval 3) *DXS95* (probe pXG7c, Davatelis *et al*, 1985) is the only polymorphic marker in this interval and, thus, is probably the most closely linked diagnostic marker of TCD available to date

These data provide a framework for the construction of a contiguous molecular map of this chromosome segment Future studies will concentrate on the molecular characterization of deletions in patients with classical, i e , nonsyndromic, TCD using chromosomal walking and jumping techniques As judged from the clinical phenotype of these individuals, the TCD gene may be the only functional gene in these deletions. If true, this should considerably facilitate its isolation

ACKNOWLEDGMENTS

The authors are indebted to Drs G A Bruns, D Drayna, M Hofker, T A Kruse, J L Mandel, D Page, K Smith, P Szabo, J Weissenbach, and B N White for generously supplying DNA probes We thank U Francke, H Lehrach, M Mikkelsen, P L Pearson, R A Pfeiffer, and A F Wright for the establishing or sharing of cell lines Furthermore, we thank F Oerlemans for technical assistance in cell culturing This work was supported by Grant 900 504 064 of the Dutch "Stichting voor Medisch Onderzoek en Gezond heidszorg" (Medigon NWO)

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Chapter 4

Deletion of the DXS165 locus in patients with classical choroideremia

Clin. Genet. 32:421-423,1987

Rapid short communication

Deletion of the DXS165 locus in patients with classical Choroideremia

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Summary

Using various probes from the Xq21 region which is known to carry the choroideremia (tapetochoroidal dystrophy, TCD) locus, we have screened the DNAs from eight unrelated male choroideremia patients for microdeletions. In two of these patients, but not in any of 45 males tested as controls, lack of hybridization signals with probe p1bD5 suggested a deletion encompassing the DXS165 locus and (part of) the TCD gene. Absence of additional clinical features in these patients and the fact that two closely linked, and probably flanking, TCD markers (DXYS1 and DXS72) are not deleted may indicate that the physical distance between the DXS165 locus and the TCD gene is small.

Introduction

The molecular defect underlying TCD is not known but recent studies have indicated close linkage with the polymorphic loci DXYS1, DXYS12, PGK1, and DXS72 (Sankila et al. 1987; Lesko et al. 1987) all of which are located in the Xq13-q21 region. Moreover, several patients with small deletions of band Xq21 have been described where TCD was associated with mental retardation (MR)(Hodgson et al. 1987), MR, cleft lip & palate and agenesis of the corpus callosum (Rosenberg et al. 1986) or with MR and deafness (Lesko et al. 1987). These observations indicate that the

TCD gene is located in the Xq21.1-q21.33 region.

As part of an ongoing study aiming at the detection of submicroscopic deletions in clinically complex X-linked syndromes we have regionally assigned 15 random DNA probes to this chromosome segment (Cremers et al. 1987a,b; unpublished results). Because of the conspicuous clustering of male-viable deletions in this region we speculated that sizeable deletions might also be found in patients where TCD is the only clinical feature. Therefore, we have screened DNA from unrelated TCD patients with several probes mapping in the relevant region of band Xq21. With one of these, deletions were found in two out of eight patients with typical TCD.

Material and Methods

In the 8 patients with TCD, blood was collected, DNA prepared and banked by the RP Center Münster for Medical Care and Research, a collaborative institution of the University of Münster and the German Retinitis Pigmentosa Association (Pawlowitzki & Brunsmann 1987). The diagnosis had been established by detailed ophthalmological examination. For patients 3.5 and 7.6, clinical findings have been reported (Hammerstein & Bohm 1985; Diekstall & Demeler 1986). DNA was isolated according to standard protocols and digested with restriction endonucleases EcoRI or TaqI. Methods for electrophoresis, Southern blotting, probe labeling and autoradiography have been described previously (Cremers et al. 1987a).

The following probes were employed: pDP34 (DXYS1), pX65H7 (DXS72), pX20R42 (DXS73), pX104f, p722 (DXS110), p784 (DXS121), pPA20 (DXS214), pXG7c (DXS95), pXG8b, and p1bD5 (DXS165). Probes pX104f and pXG8b were obtained from Drs. B.N. White and P.Szabo, respectively. All other probes have been described elsewhere (Cremers et al. 1987a; Goodfellow et al. 1985).

Results and Discussion

With 9 out of 10 probes that had been assigned to the relevant segment of band Xq21, and with probe pL2.98 (DXS56) which maps outside this region (Xq12-q13; unpublished results), normal hybridization patterns were

observed in all individuals tested. In contrast, probe p1bD5 (DXS165) reproducibly failed to detect homologous sequences in the DNA of two out of eight TCD patients studied (patients 3.5 and 7.6; see figure 1). To rule out the possibility that probe p1bD5 detects a deletion polymorphism, this probe was employed to screen genomic DNA from 45 unrelated male controls. Consistently, normal hybridization patterns were observed. This renders a deletion polymorphism very unlikely and strongly argues for the deletions being the primary cause of TCD in these patients.

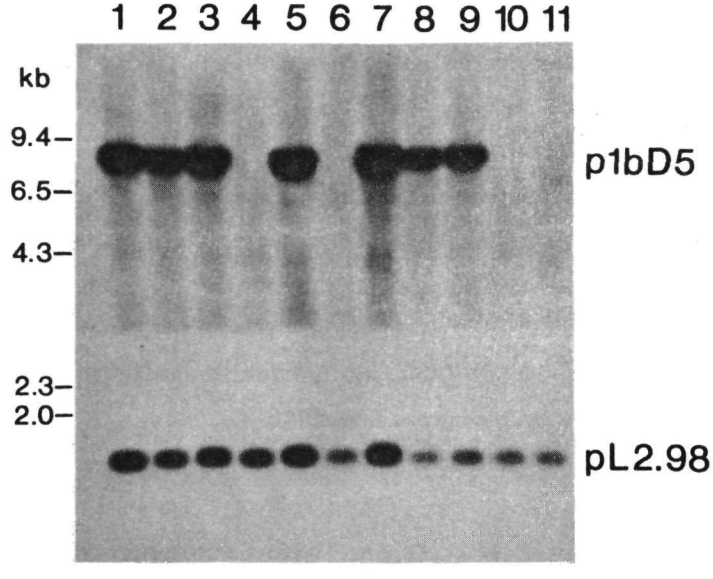

Fig. 1 p1bD5 (DXS165) hybridization signals in 7 patients with typical TCD (individuals 2.1, 3.5, 5.1, 7.6, 9.2, 10.1, 13.2; lanes 3 to 9), 2 patients with visible deletions in the Xq21 band (lanes 10 and 11), and female and male controls (lanes 1 and 2). pL2.98 (DXS56) signals are shown as internal reference.

To our knowledge, this is the first time that interstitial deletions have been detected in patients where TCD was not associated with additional genetic defects. It is noteworthy that both deletions do not include the DXS72 and DXYS1 loci which show close linkage with TCD and probably flank the TCD locus (Lesko et al. 1987). This finding suggests that the physical distance between the DXS165 locus and the TCD locus may be very small. If so, this should be a great asset for "reverse genetics" strategies aiming at the identification and isolation of the TCD gene.

For the molecular characterization of the two deletions found in this study, field inversion gel electrophoreses (FIGE; Carle et al. 1986) should be particularly helpful, and systematic FIGE screening of further TCD patients should soon clarify the role of deletions in the etiology of this disorder.

Acknowledgements

The authors are indebted to Drs. M.H.Hofker, T.A.Kruse, D.C.Page, K.Smith, P.Szabo, and B.N.White for generously supplying DNA probes, and to K.Rüther for valuable help during the RP field study which was carried out by the RP-center Münster. This work was supported by Medigon (grant no. 900-504-064), the "Aktion Sorgenkind" (grant HG-132) to I.H.P., and by the D.F.G., grant Ro389/13-3.

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Chapter 5

Chromosomal jumping from the DXS165 locus allows molecular characterization of four microdeletions and a *de novo* chromosome X/13 translocation associated with choroideremia

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Proc. Natl. Acad. Sci. USA 86:7510-7514,1989

Chromosomal jumping from the *DXS165* locus allows molecular characterization of four microdeletions and a *de novo* chromosome X/13 translocation associated with choroideremia

(retinal dystrophy/X chromosome/gene mapping)

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Communicated by Victor A McKusick July 10, 1989

ABSTRACT **Choroideremia (tapeto-choroidal dystrophy, TCD), an X chromosome-linked disorder of retina and choroid, causes progressive nightblindness and central blindness in affected males by the third to fourth decade of life. Recently, we have been able to map the *TCD* gene to a small region of overlap between five different, male-viable Xq21 deletions that were found in patients with TCD and other clinical features. Two families were identified in which classical, nonsyndromic TCD is associated with small interstitial deletions that are only detectable with probe p1bD5 (*DXS165*). To characterize these and two other deletions that were identified more recently, we have used the chromosome walking and jumping techniques to generate a set of five chromosomal-jumping clones flanking the *DXS165* locus at various distances. With these clones, we could localize four of the eight deletion endpoints and the breakpoint on the X chromosome of a female with a *de novo* X/13 translocation and choroideremia. These studies assign the *TCD* gene, or part of it, to a DNA segment of only 15–20 kilobases.**

Hereditary dystrophies of the retina and the choroid constitute a clinically heterogeneous group of Mendelian disorders that play an important role in the etiology of blindness in man Individual diseases of this group have also been shown to be genetically heterogeneous For retinitis pigmentosa (RP), autosomal dominant and recessive forms are known, and at least two distinct *RP* genes have been mapped to different sites on the short arm of the X chromosome (1–3) The gene defect underlying choroideremia, another member of this group, has been assigned to Xq13–q21 because of its tight linkage with various polymorphic probes from this region (4–6) Choroideremia is characterized by progressive degeneration of both retina and choroid leading to blindness by the third or fourth decade of life (7–9) Therefore, tapeto-choroidal dystrophy (TCD) is a more appropriate designation for this disorder Female carriers, although normally asymptomatic, show characteristic patchy changes in the retinal pigmented epithelium that reflect random X chromosome inactivation

To elucidate the molecular and, eventually, the biochemical defect underlying this disorder, we have set out to isolate the *TCD* gene by means of reverse-genetics strategies—i e , by making use of its known chromosomal location In general, very precise physical mapping, more accurate than can be achieved with linked DNA markers alone, is a prerequisite for the successful application of this strategy Physical fine mapping of probes depends on the availability of chromosomal aberrations such as translocations, duplications, and deletions Indeed, translocations and microdeletions were instrumental for the cloning of all four human genes that have been isolated in this way (10–13)

TCD and other clinical features have been described in various patients with cytogenetically visible or submicroscopic deletions spanning the Xq21 band (14–16) To determine the location of the *TCD* locus more precisely, we have characterized several of these deletions with anonymous cloned DNA sequences from proximal Xq (17) This has enabled us to divide the Xq21 band into seven different intervals and to assign the *TCD* locus to interval 3 spanning the probes pJL8 (*DXS233*), p1bD5, (*DXS165*), and pXG7c (*DXS95*) (18) Molecular characterization of patients with classical, nonsyndromic TCD revealed several additional deletions, part of which was confined to the *DXS165* locus (ref 19, and unpublished data) This suggested to us that the distance between this locus and the *TCD* gene might be small enough to be bridged by genomic walking and jumping experiments

Chromosome jumping is based on the circularization of long genomic DNA fragments and subsequent cloning of the junction fragments of these circles (20–27) In this way, it is possible to circumvent insert-size constraints of conventional cloning vectors, which limit the distance that can be covered by individual chromosome walking steps Iterative screening of a previously described chromosomal-jumping library (21) has enabled us to isolate various new DNA sequences in the vicinity of the *DXS165* locus and to determine the size of a DNA segment that is spanned by four different deletions detected in TCD patients In addition, we used these probes to map the X chromosomal breakpoint of a *de novo* X/13 translocation that has been described in a female with typical signs of choroideremia (28).

MATERIALS AND METHODS

TCD Patients. For the male patients 3 5, 7 6, 25 6, and LGL1134, the diagnosis of classical TCD had been established by detailed ophthalmologic examination (see, for instance, refs 29 and 30) A female patient showed mild choroideremia and infertility secondary to premature ovarian failure (28) By using trypsin-Giemsa staining she was karyotyped as 46,X,t(X,13)(q21 2,p12) Replication studies with

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Abbreviations TCD, choroideremia (tapeto-choroidal dystrophy), RP, retinitis pigmentosa

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BrdUrd showed that the normal X was inactivated Somatic cell hybrids formed between skin fibroblasts from this patient and mouse A9 fibroblasts yielded a mouse–human hybrid (CIII-1), which contained the derivative der(13) chromosome but not the reciprocal der(X) chromosome

Southern Blot Analysis. Chromosomal DNA was isolated as described elsewhere (31) with minor modifications DNA (10 μg) was digested with the appropriate restriction enzyme, and fragments were resolved by agarose gel electrophoresis and blotted onto GeneScreen*Plus* (NEN) membranes as described (18) Radioactive probes were prepared by random oligonucleotide priming (32, 33) Insert DNAs were electrophoretically separated from the plasmid vector and isolated in low gelling-temperature agarose Prior to hybridization, probes containing weak repetitive sequences (low-copy probes, p1bD5, p1bD5 I, pJ15 II, and pJ60) were preassociated with sonicated total human DNA as described (17, 34) Details of (pre)hybridization and washing of filters have been published (35)

Libraries. The construction of the 100-kilobase (kb) human chromosomal-jumping library has been described elsewhere (21) Approximately 2 5 × 10^6 chromosomal jumping clones ("jump clones") of the amplified library (which, before amplification, comprised about 1 5 × 10^6 independent clones) were plated on the bacterial host MC1061 (supF^-) at a density of 100,000 plaques per 130-mm plate Plating was performed in 0 7% top-agarose/Luria broth supplemented with 30% (vol/vol) glycerol for long term storage as described by Klinman and Cohen (36) Plaques were lifted on Hybond-N membranes (Amersham) and subsequently screened as recommended by the manufacturer The filters were hybridized three times with a combination of four probes Master plates were stored at −70°C Phage containing jump inserts were recovered from the master plates by drilling with a cut-off plastic tip at −20°C (36), and DNAs were purified by standard methods (37)

A phage λ EMBL 3 genomic library constructed from DNA of a human chronic myelogenous leukemia patient was provided by G Grosveld (Erasmus University, Rotterdam), 5 × 10^5 plaque-forming units were plated on *Escherichia coli* LE392 and screened as described above for the chromosomal jumping library A c2RB cosmid library was constructed from a cell line containing four X chromosomes (GM1202, 49, XXXXY) Independent clones (5 × 10^5) were plated and screened as described elsewhere (38)

Subcloning and Mapping of Inserts. Phage DNA was digested with *Eco*RI, purified by phenol and chloroform extraction, and directly cloned into *Eco*RI-cleaved, phosphatase-treated pGEM vectors or ligated into *Eco*RI-cut, dephosphorylated phage λZAPII vector arms (Stratagene) The manufacturer's excision protocol was used to convert λZAPII constructs into plasmid (Bluescript) clones Restriction maps of the inserts were generated by digestion with *Eco*RI, *Ava* I (which cuts in the middle of the *supF* gene), and *Eco*RI/*Ava* I, followed by Southern blotting and hybridization with *supF* and the starting clone

RESULTS

Identification of a Third Deletion. In a previous report we showed that probe p1bD5 (*DXS165*) detected two deletions (patients 3 5 and 7 6) among 8 patients with classical TCD (19) Screening of an additional 22 TCD patients resulted in the identification of another deletion (patient 25 6) that not only spans p1bD5 (see Fig 3*c*), but also pXG7c (*DXS95*), pXG8b, p722 (*DXS110*), pDP34 (*DXYS1*), and p47b (*DXYS5*) (data not shown) Therefore, the deletion in patient 25 6 encompasses part of interval 3, the entire interval 4, and part of interval 5 of the Xq21 band (18, 39)

Chromosomal Walking and Jumping. Employing p1bD5 as a probe on a human genomic cosmid library, clone c237 was isolated, which contained 36 kb of insert DNA (Fig 1) Single- or low-copy fragments (p1bD5-I to -IV) were subcloned, gel-purified, labeled, and hybridized to nylon filters containing *Eco*RI-digested DNA of patients 3 5, 7 6, and 25 6 and a male control In contrast to p1bD5, p1bD5-IV detects homologous sequences in the DNA of patient 7 6 (Fig 3*b*, lane 2) Therefore, one end-point of the deletion in patient 7 6 must be located between p1bD5 IV and p1bD5 We have used this breakpoint as a specific marker for the presence or absence of a deletion in females at risk for carrying the TCD defect (39) For chromosomal jumping from the *DXS165* locus, we used p1bD5 and p1bD5 IV as starting points

Screening the 100-kb human chromosomal jumping library with p1bD5-IV resulted in the isolation of one jump clone, λJ36 (Fig 2) The insert on this phage (7 5 kb *Eco*RI frag-

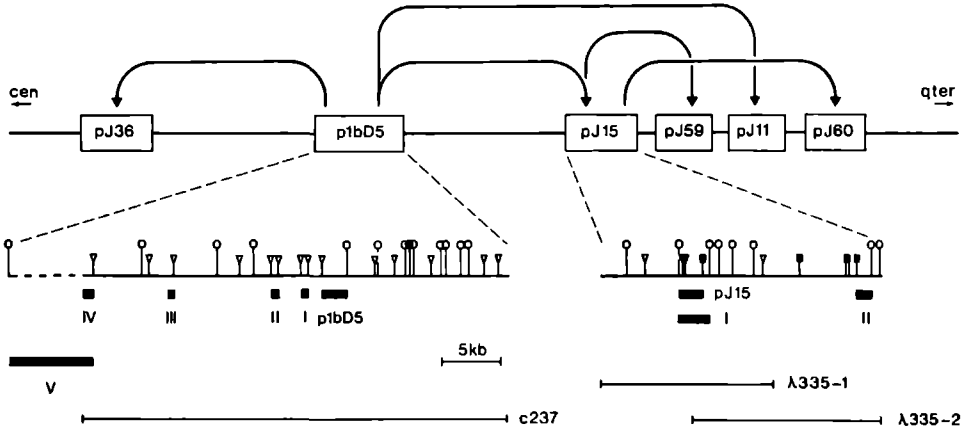

FIG 1 Chromosomal walking and jumping centromeric and telomeric of the *DXS165* (p1bD5) locus (*Upper*) The relative positions of the jump clones are based on the position of restriction sites within the jump clones (Fig 2), on Southern analysis of a deletion panel (Fig 3), and on Southern analysis of normal DNA (*Lower*) Restriction maps of the genomic clones c237 and λ335 1 and 2 The *Eco*RI (○) and *Hin*dIII (▽) sites are indicated for both c237 and the λ335 clones *Bgl* II sites (■) are only given for the λ335 clones p1bD5-V represents a sequence present in λJ36, which partly overlaps p1bD5 IV

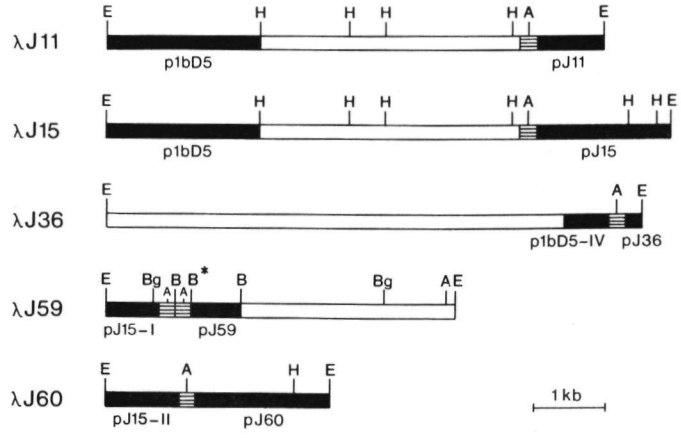

FIG. 2. Restriction maps of the phage λ inserts isolated from the chromosomal-jumping library. The *Eco*RI, *Hin*dIII, and *Ava* I sites are indicated in all inserts. For λJ59, we also indicated the *Bam*HI and *Bgl* II sites. The black boxes represent single- or low-copy sequences of the starting clones [at the left side of the *sup*F gene(s)] and the new jump clones (at the right side), whereas hatched boxes represent the *sup*F genes with their internal *Ava* I sites. The asterisk indicated in the restriction map of λJ59 indicates a *Bam*HI site that might be created in the cloning procedure. The *sup*F gene was originally flanked by *Bam*HI sites, and the genomic fragments, by *Sau*3AI sites (21).

ment; Fig. 2) consists almost entirely of a sequence (7.15-kb *Eco*RI/*Ava* I fragment) that spans part of p1bD5-IV and extends to an *Eco*RI site situated outside of c237 (denoted p1bD5-V, see Fig. 1). The remaining part of this insert, designated pJ36 (0.35-kb *Eco*RI/*Ava* I fragment), was used as a probe to screen a Southern blot carrying DNA from the three deletion patients. As shown in Fig. 3*a*, pJ36 is located outside the 7.6 and 25.6 deletions but still inside the 3.5 deletion. This indicates that with this jump we have crossed an endpoint of deletion 25.6. Since the 25.6 deletion encompasses several probes that are located distal to p1bD5, this breakpoint must be proximal to p1bD5. At the same time, this proves that the *TCD* gene must be located telomeric to the 7.6 deletion breakpoint.

With probe p1bD5, two different jump clones, λJ11 and λJ15, could be isolated (Fig. 2). The end fragments of both clones, pJ11 (1.05-kb *Eco*RI/*Ava* I fragment) and pJ15 (2.0-kb *Eco*RI/*Ava* I fragment), are devoid of repetitive sequences and are located within all three deletions (for pJ15, see Fig. 3*d*). To generate starting probes for further jumps in the telomeric direction, both single-copy probes were used to isolate corresponding sequences from a λ phage human genomic library. With probe pJ15, two clones with overlapping inserts were detected (λ335-1 and -2), spanning a total of 23 kb (Fig. 1). The orientation of these clones was deduced from the location of two *Hin*dIII sites situated within 500 base pairs (bp) of the *Eco*RI site of pJ15 (Figs. 1 and 2). In the λ335 inserts, two useful sequences were identified, a unique copy probe spanning pJ15, pJ15-I (2.5-kb *Eco*RI fragment), and pJ15-II—a low-copy probe (1.3-kb *Bgl* II/*Eco*RI fragment) located just centromeric from an *Eco*RI site (Fig. 1).

Screening the chromosomal-jumping library with pJ15-I yielded a λ phage (λJ59) that contained a fragment of 3.9-kb next to the pJ15-I sequences. From this fragment, a single-copy sequence (pJ59; 0.7-kb *Bam*HI fragment) was derived and subsequently used to screen the three deletions. Absence

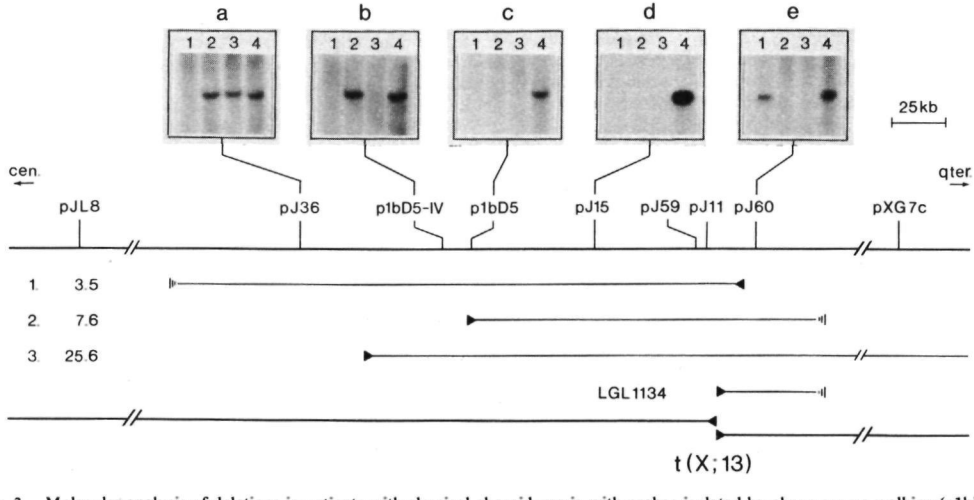

FIG. 3. Molecular analysis of deletions in patients with classical choroideremia with probes isolated by chromosome walking (p1bD5-IV) and jumping (pJ36, pJ15, and pJ60). (*Upper*) (*a–e*) *Eco*RI-digested DNA of patients 3.5 (lanes 1), 7.6 (lanes 2), and 25.6 (lanes 3) and a male control (lanes 4). The presence of comparable amounts of DNA in each lane was shown by using a probe located outside this region (pL2.98; Xq13; ref. 17). (*Lower*) Deleted regions of patients 3.5, 7.6, 25.6, and LGL1134 are depicted between arrowheads. The position of the centromeric deletion endpoint in patient 3.5 and the telomeric deletion endpoints in patients 7.6 and LGL1134 are unknown. The telomeric deletion endpoint in patient 25.6 is located in interval 5 of Xq21 (18, 39). The location of the X chromosomal breakpoint of a X/13 translocation in a female with choroideremia is shown at the bottom.

of hybridization signals revealed that pJ59, like pJ11, pJ15, and p1bD5, maps to the DNA segment that is deleted in all three patients. The sequences in λJ59 that are homologous to pJ15-I (0.7-kb *Eco*RI/*Ava* I fragment) contained a *Bgl* II site but no *Hin*dIII sites (Fig. 2), indicating a jump in the telomeric direction as depicted in Figs. 1 and 3.

Screening of the chromosomal-jumping library with pJ15-II yielded a λ phage (λJ60; Fig. 2) that contained a low-copy sequence, pJ60 (1.7-kb *Eco*RI/*Ava* I fragment). This sequence is located inside the 7.6 and 25.6 deletions but distal to the 3.5 deletion (Fig. 3*e*). Thus, this jump has provided us with the distal demarcation of the segment that all three deletions have in common.

The relative positions of pJ11 and pJ59 with respect to pJ60 were deduced from conventional Southern analysis. pJ59 and pJ11 hybridize to the same 16-kb *Hin*dIII fragment, whereas pJ11 and pJ60 are both located on a *Bam*HI fragment of approximately 36 kb (unpublished data). These results suggest the order Xcen-pJ59-pJ11-pJ60-pter. Field inversion gel electrophoresis analysis indicated that all jump clones, as well as *DXS165*, are located on a 400-kb *Sal* I and a 625-kb *Sfi* I fragment (unpublished data).

With these jump clones as probes, a fourth patient with TCD and a microdeletion was found. This deletion (LGL1134) spans pJ60, but none of the other jump clones nor the *DXS95* (pXG7c) locus (for pJ11, pJ15, and pJ60, see Fig. 4*a*). The *Hin*dIII fragment detected by pJ11 in DNA of patient LGL1134 is slightly smaller than that of a male control, which indicates that the centromeric deletion endpoint is located within this 16-kb *Hin*dIII fragment. Preliminary restriction mapping data indicate that the deletions in patients 3.5 and LGL1134 overlap a DNA segment of approximately 15–20 kb.

Mapping of the Translocation Breakpoint. DNA of the cell hybrid CIII-1 and a male control was analyzed with all jump clones from this region, and the anonymous probes p1bD5 (*DXS165*), pXG7c (*DXS95*), and p22.33 (*DXS11*), which maps further telomeric on Xq (40). As shown in Fig. 4*b*, probes p1bD5, pJ15, and pJ11 fail to hybridize to DNA from the CIII-1 hybrid, whereas sequences pJ60, pXG7c (not shown), and p22.33 are present on the der(13) chromosome of this cell line. Therefore, the X chromosomal breakpoint of this translocation can be positioned between pJ11 and pJ60, within or just proximal to the DNA segment that is deleted in the four patients described above (Fig. 3).

DISCUSSION

So far, there is only a limited number of reports dealing with the successful application of chromosome-jumping techniques (21, 23–25, 27). In this study, we made use of a 100-kb human chromosomal-jumping library that had been used previously to study the chromosomal region carrying the cystic fibrosis locus (21) and the Duchenne gene (24). One of the advantages of this library is that any sequence, unless located on a *Eco*RI fragment containing no *Sau*3AI restriction sites, can be used as a starting clone. Several different probes from one locus can be tested, and directional jumping can be achieved by using probes that are optimally positioned with respect to *Eco*RI sites (20, 26). Furthermore, the broad size range of the jumps (40–120 kb) results in "scanning" of neighboring genomic regions. Because of the relatively small average insert size (about 5 kb), approximately 2×10^6 independent phage must be plated to achieve a 95% coverage of the genome. To avoid repeated phage plating and lifting, we applied a long-term storage procedure for plated phage (38) in combination with plaque-lifting on nylon filters that can be rehybridized. Phage plated in this way remained viable for at least 1 year.

We used the *DXS165* locus as a starting point for jumps in the centromeric and the telomeric direction. The resulting jump clones were positioned with the help of four, partially overlapping, deletions present in the genome of classical TCD patients. The orientation of the probe cluster depicted in Figs. 1 and 3 was established by jump clone pJ36, which was located outside the 7.6 and 25.6 deletions, but inside the 3.5 deletion. As the 25.6 deletion does not only span *DXS165* (p1bD5) but also five loci that map telomeric to this marker, the order of loci in interval 3 (18) is firmly established as Xcen-*DXS233-DXS165-DXS95*-qter.

In most experiments, the direction of jumping could be predetermined by choosing starting clones that were located next to *Eco*RI sites (Fig. 1). The position of the jump clones was confirmed by Southern blot analysis of a deletion panel (Fig. 3). Jump clone pJ60 is located inside the 7.6 and 25.6 deletions but outside the 3.5 deletion. This means that we have jumped from one proximal deletion endpoint (patient 7.6) across another one that forms the distal demarcation of the deletion in patient 3.5. As one jump (pJ11) was almost sufficient to bridge this distance, we estimate that the region of overlap between these three deletions does not exceed 150 kb. The broad size range of the jumps performed with this

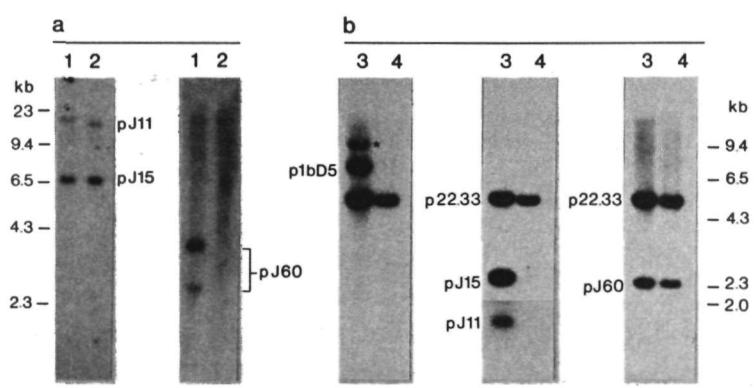

FIG. 4. (*a*) Southern blot analysis of *Hin*dIII-digested DNA of a control male (lanes 1) and patient LGL1134 (lanes 2). (*Left*) Southern blot hybridized with pJ11 and pJ15. (*Right*) The blot was stripped and rehybridized with pJ60, which detects *Hin*dIII fragments of 2.8 and 3.9 kb (see Fig. 1). (*b*) Southern blot analysis of *Eco*RI-digested DNA of a control male (lanes 3) and cell line CIII-1 (lanes 4). The six lanes were run in parallel and blotted as described. (*Center*) The lower part was exposed for a longer time to enhance the signal of probe pJ11 compared to pJ15. (*Left*) The asterisk indicates a second *Eco*RI fragment detected by p1bD5, which most likely is due to incomplete digestion of the control DNA.

library is illustrated by the fact that two successive jumps, pJ15 and pJ59, have resulted in bridging a distance comparable to that of one jump (pJ11)

Screening of additional TCD patients revealed another deletion (LGL1134), which is only detectable with the pJ60 probe Preliminary data from Southern blot analyses indicate that the deletions in patients 3 5 and LGL1134 share a DNA segment of 15–20 kb that maps between pJ11 and pJ60 Furthermore, we were able to localize a translocation breakpoint, which presumably disrupts the *TCD* gene, between these two markers

The size of the *TCD* microdeletions can only be estimated indirectly The deletion in patient 25 6 contains six anonymous DNA loci and is estimated to span >5000 kb The jump clones depicted in Figs 1 and 3 are all located on a 625-kb *Sfi* I fragment and a 400-kb *Sal* I fragment, previously shown to contain the *DXS165* locus (18) For patients 3 5, 7 6, and LGL1134, deletion sizes cannot be determined with certainty, as the position of their centromeric (patient 3 5) or telomeric endpoints (patients 7 6 and LGL1134) relative to *Sal* I and *Sfi* I sites cannot be established

In view of the apparent causal relationship between the microdeletions and the TCD phenotype of patients 3 5, 7 6, 25 6, and LGL1134, our studies have provided convincing evidence for (part of) the *TCD* gene to be located on a 15- to 20-kb segment between pJ11 and pJ60 This is corroborated by the fact that a translocation breakpoint associated with the TCD phenotype in a female is also located within or very close to this DNA segment

We are indebted to Dr G Grosveld for sharing a genomic library and Drs R L Nussbaum and P Szabo for supplying DNA probes This work was supported by Grant 900-504-064 from the Dutch 'Stichting voor Medisch Onderzoek en Gezondheidszorg" (Medigon NWO) and National Institutes of Health Grant GM34960 to F S C , who is also an Associate Investigator of the Howard Hughes Medical Institute

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Chapter 6

Derivation of clones from the choroideremia locus by preparative field inversion gel electrophoresis

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Nucleic Acids Res. 18:725-731,1990

Derivation of clones from the choroideremia locus by preparative field inversion gel electrophoresis

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Received December 14, 1989, Revised and Accepted January 17, 1990

ABSTRACT

By making use of preparative field inversion gel electrophoresis, we have constructed a lambdaZAP library that is highly enriched for sequences from the choroideremia locus. *In vivo* excision of pBluescript SK(−) constructs from lambdaZAP obviates the subcloning of DNA inserts and allows for rapid processing of several hundred recombinants. From a 625 kb *Sfi*I fragment we isolated 7 clones that were physically mapped using microdeletions associated with the disease. One of these clones is located within, or just telomeric to, the choroideremia gene and detects two restriction fragment length polymorphisms (RFLPs). Another clone detects a RFLP which maps centromeric to the disease locus. Together these probes should improve the reliability of linkage analysis in choroideremia families and should pave the way for the isolation of the choroideremia gene.

INTRODUCTION

Choroideremia is an X-linked chorioretinal dystrophy in which affected males suffer from progressive night blindness and visual field constriction, leading to blindness by the third to fourth decade of life (1–4) The gene locus for choroideremia (tapetochoroidal dystrophy TCD) has been assigned to Xq21 by means of genetic linkage and physical mapping studies (5–12) Characterization of various deletions associated with clinically complex TCD enabled us to refine the assignment of the TCD locus to a small segment in Xq21 (13) Deletions encompassing only the DXS165 locus were found in patients with classical TCD (14) Chromosomal jumping from the DXS165 locus allowed us to define the minimal region of overlap between four such microdeletions, and to map a *de novo* X,13 translocation disrupting the TCD gene in a female TCD patient (15) These studies indicated that at least part of the TCD gene is located on a 625 kb *Sfi*I fragment.

In this paper we describe the use of preparative FIGE to establish a library highly enriched for sequences from a 625 kb *Sfi*I fragment which contains part of the TCD gene locus

MATERIALS AND METHODS

Preparative Field Inversion Gel Electrophoresis

The somatic cell hybrid 578 contains a Chinese hamster genome (parent Wg3-h) and a single human X chromosome Cells were grown under HAT selection and embedded at a concentration of 7.5×10^6 cells/ml in 0.6% low-gelling-temperature (LGT) agarose (0.3×10^6 cells/block) as described (16,17) 21 blocks were preincubated overnight at 4°C in 5 ml *Sfi*I digestion buffer as recommended by the manufacturer Digestion was performed for 3 h at 50°C in 1.2 ml buffer to which 2 mM spermidine, 100 μg/ml BSA, and 200 U *Sfi*I were added Thereafter, 100 U of enzyme was added, and digestion was allowed to continue for another 3 h to assure completeness

The preparative gel, sized $0.6\times17\times21$ cm, consisted of 1% (w/v) LGT agarose in 0.5×TBE buffer (16) 20 blocks were transferred to a 2 mm-wide and 9 cm-long slot. One block was loaded into a separate slot (the 'testlane'), and *Saccharomyces cerevisiae* chromosomes were used as size markers Field inversion gel electrophoresis (FIGE) was performed for 23 h at 6°C and 160 V Switching intervals increased linearly from 6 to 60 s for the forward phase and from 2 to 20 s for the reverse phase

After electrophoresis, the lanes containing size markers and the separate test DNA were cut off, stained in 10 mM EDTA containing 1 μg/ml ethidium bromide for 1 h, destained in 10 mM EDTA for 1 h and photographed (Fig 2) DNA in the test lane was transferred to a Hybond-N membrane (Amersham) as described previously (13), rinsed in 2× SSC, allowed to air-dry, and exposed to UV-light (305 nm) for 3 min Hybridization with probe p1bD5, and washing of the membrane was performed as recommended by the manufacturer The central unstained section of the gel was sliced into 2 mm wide strips perpendicular to the running direction A small segment of each strip, corresponding to one lane of DNA, was used for dotblotting and hybridization The remaining strips were stored in 0.5 M EDTA, pH 8 at 4°C.

Dot blotting

Pieces of the LGT agarose gel corresponding to one lane of DNA from cell line 578 (volume 75–100 μl) were melted for 5 min at 95°C, and brought to 0.3–0.5 M NaOH After vortexing, the samples were incubated at 65°C for 10 min , and spotted

onto a Hybond-N membrane by means of a dot blot apparatus that had been pre-warmed to 37°C The following samples were included as controls i a 1% LGT agarose block in 0 5 TBE without DNA (Fig 2c, #50), ii 4 μg of high molecular weight DNA isolated from a male (#47), a female (#48), and a cell line containing 4 X chromosomes (LCL127, #49) The membrane was neutralized by washing in 0 5 M Tris-HCl pH 7 5/1 5 M NaCl/1 mM EDTA for 10 min , air-dried, exposed to UV light as described above, and hybridized with probe p1bD5

DNA recovery and cloning in lambdaZAP

LGT agarose strips containing DNA which yielded hybridization signals with the p1bD5 probe (#31 and #32, Fig 2c), were rinsed three times for 2 h in 10 mM Tris-HCl pH 7 5/1 mM EDTA/100 mM NaCl, melted at 65°C, and incubated overnight with 50 U agarase/ml (Calbiochem) at 37°C High molecular weight DNA was recovered by sequential phenol and chloroform extractions (both twice) and by ethanol precipitation in the presence of 0 3 M NaAc, pH 6

From each slice approximately 500 ng of DNA was recovered 800 ng of pooled DNAs from slices #31 and #32 was digested with 10 U *Eco*RI, purified, and concentrated using Geneclean™ (Bio101 Inc) DNA recovery of this step was estimated to be 40%, yielding approximately 320 ng 150 ng of purified *Eco*RI digested DNA was ligated to 2 μg of *Eco*RI digested and dephosphorylated lambdaZAP arms (Stratagene, La Jolla, CA) From 10 μl of the ligation mixture, 2 5 μl (0 5 μg) aliquots were packaged using the Promega packaging extracts, resulting in a total of $1\,6\times10^5$ pfu Screening of randomly picked phage, revealed that 75% contained an insert in the size range between 0 5 and 10 0 kb (not shown)

Identification of human clones

Recombinant lambdaZAP phage were allowed to transfect the *E coli* host strain BB4 according to recommendations of the manufacturer Approximately 3×10^4 insert-containing phage were plated on 130 mm plates at a density of 300 phage per plate Plaques were lifted onto nitrocellulose filters (Schleicher & Schuell, BA85) using routine procedures (18) Two replica filters from each master plate were probed with human DNA at a concentration of 1−3 ng/ml Labeling was performed by primed synthesis with Klenow DNA polymerase I and α-^{32}P-dCTP as described by Feinberg and Vogelstein (19,20) Methods for (pre)hybridization, washing, and exposure of filters have been described elsewhere (21)

Human plaques were isolated from the plates using sterile pasteur pipettes, diluted into SM-buffer (0 1 M NaCl/10 mM $MgSO_4$ $7H_2O$/1 M Tris-HCl pH7 5/0 01% (w/v) gelatin), and stored at 4°C LambdaZAP recombinants were transformed to plasmid clones (pBluescript) using the Stratagene lambdaZAP excision protocol Plasmid containing BB4 cells were plated on LB plates containing 50 μg/ml ampicilline, and grown overnight Colonies were individually picked and grown overnight in LB/ampicilline (50 μg/ml) medium at 37°C Plasmid DNA was isolated using the miniprep boiling-method described by Holmes and Quigley (22) Recombinant plasmids were *Eco*RI digested and inserts were separated from vector sequences by electrophoresis in 1% agarose-gels Each gel was sequentially blotted, for 25 min and 3 h respectively, to two nitrocellulose filters which were then hybridized to labeled human and hamster DNA, respectively Recombinants containing inserts that appeared to be of human origin were run on a 0 8% LGT agarose gel and the inserts were isolated for labeling and hybridization

Chromosomal localization

In order to localize the human DNA sequences, we constructed three types of blots

i Filters containing 10 μg *Eco*RI digested DNA from two patients, NP and RvD, and a control male The deletion in patient RvD spans Xq21 1-q22 1, whereas NP carries a deletion which encompasses Xq21 1-q21 33 Clinical features have been described in detail elsewhere (11,13) Both patients have complex clinical syndromes, NP shows typical signs of TCD, RvD does not

ii Filters containing 10 μg *Eco*RI digested DNA from 3 patients with classical TCD (3 5 [Fig 3, lane 1], 7 6 [lane 2], 25 6 [lane 3]), and from a male control (lane 4) Patients 3 5 and 7 6 carry submicroscopic deletions that overlap for approximately 120 kb in the relevant 625 kb *Sfi*I band, and extend to opposite directions as shown by molecular characterization (15) The deletion in patient 25 6 overlaps partly with the deletion in patient 3 5, and contains the entire segment deleted in patient 7 6 The 25 6 deletion spans 6 anonymous markers from the Xq21 band (Xq21 2-q21 31), and is estimated to encompass at least 5000 kb (unpublished data)

iii FIGE blots containing *Sfi*I and *Sal*I digested DNA from cell line LCL127 (48, XXXX) Preparation of high-molecular-weight DNA in agarose blocks, restriction enzyme analysis, FIGE electrophoresis, and blotting to Hybond-N membranes have been described elsewhere (13) Insert DNAs isolated from LGT agarose gels were labeled by primed synthesis with Klenow DNA polymerase I and α-^{32}P-dCTP, as described previously (19,20) Labeled inserts were prehybridized to sonicated human DNA (Cot = 3,600 mg×min/ml), essentially as described by Litt and White (23), to block highly and moderately repetitive sequences present in most of the probes

Blots described above were stripped following each hybridization and re-used several times

RESULTS

FIGE analysis using jumpclones

In a previous study we have reported the isolation of several jumpclones from the choroideremia area, employing probes p1bD5 and p1bD5-IV as molecular entry sites (15) These clones were hybridized to FIGE blots containing *Sfi*I, *Sal*I, or *Sfi*I/*Sal*I digested control DNA (LCL127, 48, XXXX) As shown for the most centromeric and telomeric jumpclones (pJ36 and pJ60), all clones detect a 625 kb *Sfi*I, a 400 kb *Sal*I (Fig 1a,b), and a 350 kb *Sfi*I/*Sal*I fragment (not shown) As the jumpclones flank a DNA segment that is deleted in three TCD patients, the 625 kb *Sfi*I fragment should contain at least part of the TCD gene (Fig 5)

The proximal deletion breakpoints of patients 7 6 and 25 6 map within the 625 kb *Sfi*I fragment For patient 3 5, at least the distal and possibly the proximal deletion breakpoint map within this fragment Of all jumpclones that hybridize to a 625 kb *Sfi*I fragment in normal DNA, pJ60 is the only one that yields a hybridization signal in DNA of patient 3 5 On FIGE blots of this patient, pJ60 detects a 215 kb *Sfi*I fragment (Fig 1c) Depending on whether the 3 5 deletion is completely contained

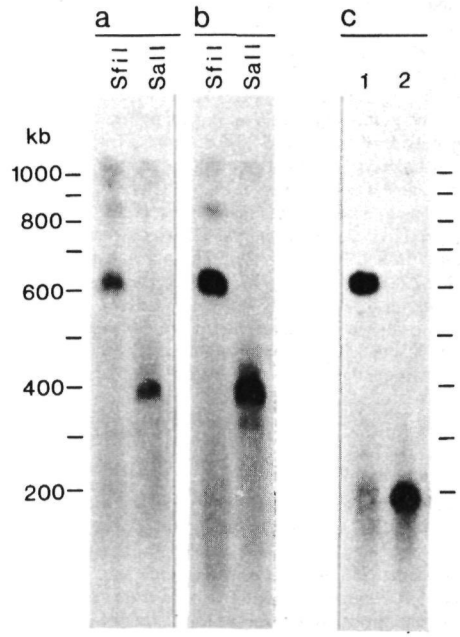

Figure 1: FIGE analysis with jumpclones, (a,b). LCL 127 DNA was digested with the enzymes indicated for each lane. (c). DNA of a control male (lane 1) and patient 3.5 (lane 2) was digested with *SfiI*. Blots were hybridized with pJ36 (a) and pJ60 (b,c). The scale shown on both sides of the panels is based on the migration of the *Saccharomyces cerevisiae* chromosomes which were employed as size markers.

within the 625 kb *SfiI* fragment, this might indicate that the size of this deletion is 410 kb.

The combined mapping results of the deletion endpoints divide the 625 kb *SfiI* fragment into 4 segments, denoted A through D (Fig. 5).

Preparative FIGE

In order to identify the agarose-gel strips that contain the 625 kb *SfiI* fragment, p1bD5 was hybridized to *SfiI* digested DNA from cell line 578 (Fig. 2a), and to a dotblot containing DNA samples from the sliced preparative gel (Fig. 2c). Clearly, slices #31 and #32 contain sequences that are homologous to probe p1bD5. DNA recovered from these slices was digested with *Eco*RI, and subcloned in lambdaZAP. We plated 3×10^4 insert-containing phage from the total library (1.6×10^5 pfu) on NZY plates, and selected 232 plaques that showed a moderate or strong signal when hybridized to human DNA. The lambdaZAP excision procedure was performed for each individual recombinant to obtain the corresponding pBluescript clone. Small amounts of plasmid DNA were isolated, and the origin of the inserts was established by hybridization with either ^{32}P-labeled human or hamster DNA. Most of the inserts of recombinant clones consisted of more than one *Eco*RI fragment, suggesting either concatenization of genomic *Eco*RI inserts during ligation, or incomplete *Eco*RI digestion of the original 625 kb *SfiI* fragment prior to cloning. Of 232 clones analysed, 153 were considered to be of human origin and selected for further analysis (Table 1). The remaining clones hybridized strongly to hamster DNA, contained no insert, or could not be transformed into Bluescript plasmids. No recombinant clones were found that contained both human and hamster DNA, which supports our assumption that inserts consisting of multiple *Eco*RI fragments are due to incomplete *Eco*RI digestion of the 625 kb *SfiI* fragment, and hence are derived from a single site on the X-chromosome.

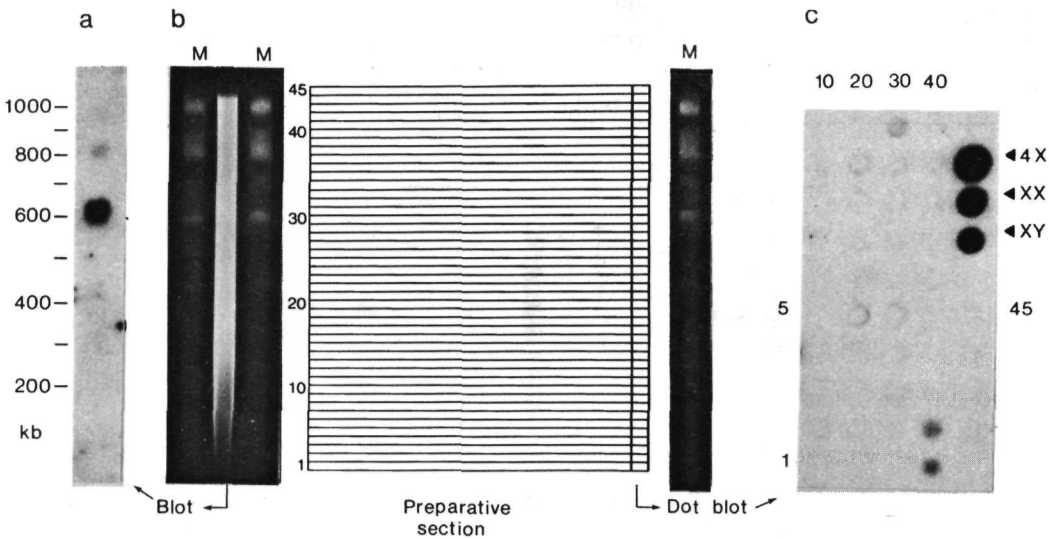

Figure 2: Isolation of the 625 kb *SfiI* band. (a) Southern blot analysis of *SfiI* digested DNA from cell line 578 which carries a single human X chromosome. (b) Dissection of the preparative FIGE gel into 45 fractions and their position relative to yeast chromosome markers (M). The second lane from the left contains ethidium bromide-stained, *SfiI* digested DNA from the 578 cell line. (c) Dot blot containing 45 DNA samples from the central preparative part, and high molecular weight DNA of control male (#47), female (#48), and a 4× cell line (LCL127; #49). Blots (a) and (c) were hybridized with probe p1bD5.

Localization of human clones

Hybridization of inserts from the 153 putative human clones to *Eco*RI digested DNA from patients RvD and NP showed that 9 clones did not yield discrete hybridization signals, 20 were of hamster origin, and 124 contained human inserts. From these, 22 clones were deleted in the DNA of both NP and RvD, and 3 clones were located only in the RvD deletion, indicating an assignment to Xq21.33-q22.1 (Table 2).

Probes located in the RvD and NP deletions were hybridized to Southern blots carrying *Eco*RI digested DNA from a healthy male and from 3 patients with typical TCD and small deletions encompassing part of the relevant 625 kb *Sfi*I fragment. 9 clones were found to be located outside all 3 deletions, 13 were assigned in at least one of these deletions (Table 2). Five of these probes, i.e. pZ25 (Fig. 3a), pZ138, pZ172, pZ381, and pZ508, could be assigned to the region deleted in patient 3.5, but were found to map outside of the 7.6 and 25.6 deletions. Thus, these probes can be positioned in interval A within or centromeric to the 625 kb *Sfi*I fragment, as depicted in Fig. 5. Clone pZ521 is located in all 3 deletions and hence, maps to interval C (see Fig. 3b). Clone pZ11 is located in interval D, which is spanned by the deletions 7.6 and 25.6 (Fig. 3c). The remaining 6 clones, i.e. pZ169, pZ228, pZ231, pZ359, pZ384, and pZ394 (Fig. 3d), are located in deletion 25.6, but not in deletions 3.5 and 7.6. There is evidence that these clones are not derived from the relevant 625 kb *Sfi*I fragment thought to carry most of the TCD gene, but from other *Sfi*I fragments of similar size, as discussed below.

Table 1: Cloning DNA from an 625 kb *Sfi*I fragment

Screening stage	No. of clones
Total recombinants tested	30.000
Human specific upon plaque screening	232
Human specific upon miniprep screening	153
X-chromosome specific signal	124
Localization in Xq21.1-q22.1	25
Assigned to 625 kb fragment	7

Table 2: Fine mapping of probes from the Xq21.1-q22.1 segment by deletion analysis

Clone(s)		RvD	NP	25.6	3.5	7.6	Assignment
pZ25	pZ138						
pZ172	pZ381	–	–	+	–	+	Xq21.2
pZ508							
pZ521		–	–	–	–	–	Xq21.2
pZ11		–	–	–	+	–	Xq21.2
pZ169	pZ228						
pZ231	pZ359	–	–	–	+	+	Xq21.2-q21.31
pZ384	pZ394						
pZ17	pZ129						
pZ167	pZ196						Xq21.1
pZ221	pZ251	–	–	+	+	+	or
pZ294	pZ614						Xq21.31-q21.33
pZ618							
pZ321	pZ527						
pZ537		–	+	+	+	+	Xq21.33-q22.1

FIGE analysis

When hybridized to blots carrying *Sfi*I and *Sal*I digested DNA from a 48,XXXX cell line, only 2 out of 17 clones previously shown to be absent in the DNA of both RvD and NP, failed to detect specific *Sfi*I bands. From the remaining 15 clones, 14 detected *Sfi*I fragments ranging in size from 600 to 650 kb, whereas one probe hybridized to a *Sfi*I fragment of 700 kb.

The five clones that were tentatively assigned to interval A detect a 625 kb as well as an 850 kb *Sfi*I fragment (see probe pZ25 in Fig. 4a and pZ172 in Fig. 4b). The 850 kb *Sfi*I band is also seen with jumpclones that are located on the relevant 625 kb *Sfi*I fragment, e.g. pJ60 (Fig. 4c). The *Sal*I fragments detected by probes from interval A are smaller than 100 kb, or cannot be discriminated from the background signal. So far, we did not find any clones that map to the 625 kb *Sfi*I fragment, but centromeric to the 3.5 deletion. The FIGE analysis strongly suggests that all five clones from interval A are located on the 625 kb *Sfi*I fragment. Hence, we cannot exclude that the proximal endpoint of deletion 3.5 maps centromeric to the 625 kb *Sfi*I fragment.

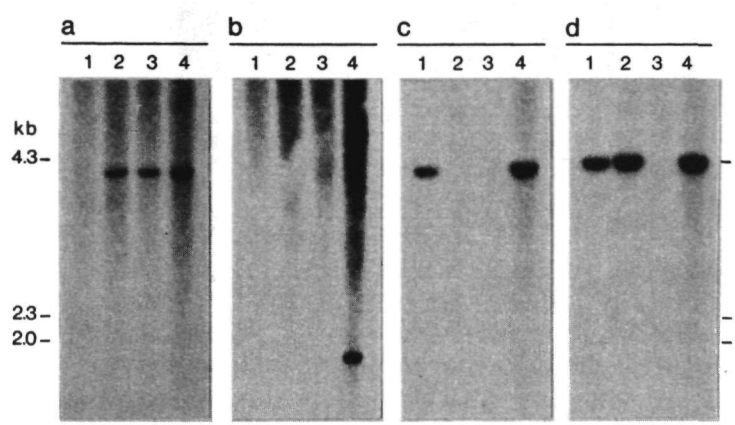

Figure 3: Southern blot analysis of *Eco*RI digested DNA of classical TCD patients 3.5 (lane 1), 7.6 (lane 2), 25.6 (lane 3), and a control male (lane 4). Blots were hybridized with pZ25 (a), pZ521 (b), pZ11 (c), and pZ394 (d).

Clone pZ521 is located in a DNA segment (interval C) that is deleted in 3 patients with classical TCD. Southern analysis of all previously isolated genomic sequences from interval C has mapped pZ521 within 2 kb of jumpclone pJ15 (15). FIGE analysis did not result in discrete hybridization signals, as pZ521 consists almost entirely of highly repetitive sequences.

Clone pZ11 consists of a single copy 1.2 kb *Eco*RI fragment (pZ11a), and a repetitive 4.2 kb *Eco*RI fragment (pZ11b). pZ11a and pZ11b are located next to eachother in the X-chromosome (unpublished data). pZ11a does not only detect the characteristic *Sfi*I bands of 625 and 850 kb, but also hybridizes to a 400 kb *Sal*I band (Fig. 4d). We have cloned 45 kb of genomic DNA spanning the jumpclones pJ59, pJ11, and pJ60, as well as the distal breakpoint of deletion 3.5. Southern analysis of these genomic clones has shown that pZ11 is not located in this DNA segment, and therefore must be situated telomeric to pJ60.

The six clones found to be deleted in patient 25.6, detect a *Sfi*I band of 615 kb, and do not hybridize to the characteristic 850 kb *Sfi*I band (Fig. 4e and 4f). Moreover, they fail to detect the typical 400 kb *Sal*I band. Taken together, this indicates that these clones are derived from a different *Sfi*I fragment in the Xq21.2-q21.31 region (Table 2).

Restriction fragment length polymorphisms

Southern analysis of DNA from TCD patients and controls revealed three new restriction fragment length polymorphisms (RFLPs). pZ11b detects variable *Eco*RI bands of 4.2 and 4.5 kb, and a constant band of 1.4 kb. Both the variable and constant bands are located in interval D of the 625 kb *Sfi*I fragment. We tested 62 X-chromosomes from unrelated individuals, and observed allele frequencies of 66% for the larger (4.5 kb), and 34% for the smaller (4.2 kb) allele. Also, pZ11b detects an *Eco*RV RFLP, with variable bands of 2.0 kb plus 5.3 kb, and 7.3 kb respectively, and a constant band of 6.7 kb. Estimated allele frequencies, were 53% for the 7.3 kb band, and 47% for the 2.0/5.3 kb bands.

pZ172 detects variable *Eco*RI bands of 5.5 and 7.0 kb, and a constant band of 3.0 kb. The polymorphic locus recognised by pZ172 maps to interval A of the 625 kb *Sfi*I fragment; the constant band is located outside the Xq21 region. We tested a total of 43 X-chromosomes from unrelated individuals and observed allele frequencies of 40% for the larger (7.0 kb) and 60% for the smaller (5.5 kb) allele. Both for pZ11b and pZ172, no other enzymes were tested for RFLPs.

DISCUSSION

The molecular and biochemical defects underlying several genetic disorders have been elucidated by means of the so called 'reverse genetics' approach (24–27). One of the crucial, and most tedious, steps in this approach is the generation of a large number of probes from the relevant DNA region, which are then used for the isolation of an overlapping set of lambda or cosmid clones (27–37). An elegant, but seldomly used, technique makes use of preparative FIGE/PFGE (pulsed field gel electrophoresis), to enrich for sequences from a well defined, large DNA fragment which encompasses the disease gene (38,39).

In a previous study (15), we described the use of a human jumping library that enabled us to isolate several DNA markers from the vicinity of the TCD gene in a directional manner. The minimal region of overlap between several deletions associated with TCD could be positioned on a large *Sfi*I fragment of 625 kb. Thus, two important prerequisites were fulfilled for the application of preparative FIGE. First, enrichment of genomic sequences from the TCD gene region was facilitated by the fact that the fragment of interest is located outside the bulk of the *Sfi*I genomic fragments (100–500 kb). Second, the identification of relevant probes from this fragment could be rapidly determined

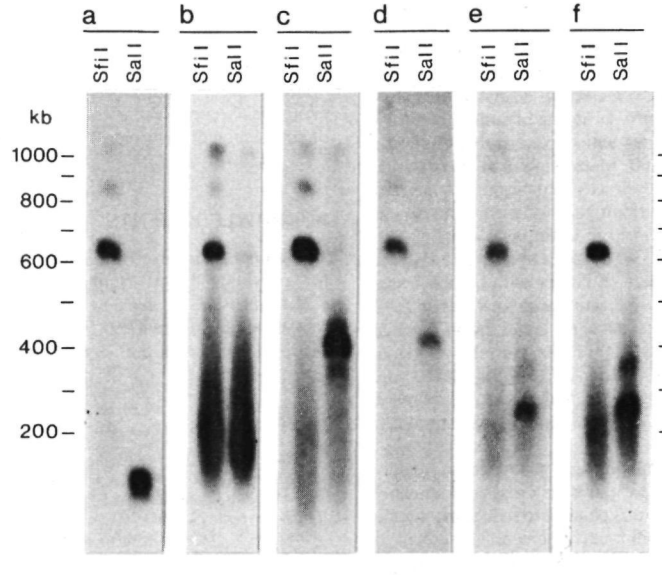

Figure 4: FIGE analysis with probes pZ25 (a), pZ172 (b), pJ60 (c), pZ11 (d), pZ169 (e), and pZ394 (f). LCL127 DNA was digested with *Sfi*I or *Sal*I as indicated for each lane. The scale on the left is based on the migration of yeast chromosomes as given in Fig. 1.

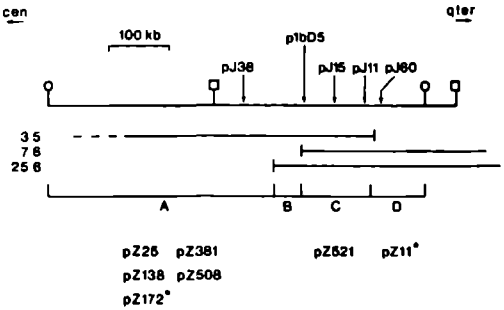

Figure 5: Schematic drawing of the 625 kb *SfiI* fragment which is flanked by open circles *SalI* restriction sites are indicated by open squares. Through analysis of deletions 3 5, 7 6, and 25 6, the 625 kb fragment could be subdivided into four regions i e A, B, C, and D The preparative FIGE clones could be assigned to several of these intervals by making use of conventional Southern analysis, and analytical FIGE The centromeric deletion endpoint of patient 3 5 has not yet been mapped, because so far, no clone has been identified that is located centromeric to the 3 5 deletion, and inside the relevant 625 kb *SfiI* fragment. Asterisks indicate polymorphic clones.

by Southern analysis of three small deletions encompassing part of the TCD gene. Still, despite the considerable enrichment achieved through selection and cloning of this fragment, large numbers of human clones had to be analysed to obtain a reasonable number of starting points from the fragment of interest. This illustrates the crucial importance of the cloning system employed. The cloning efficiency should be as high as possible because of the limited amount of DNA that can be recovered from LGT-agarose slices. In addition, human DNA sequences often represent only a minor fraction of the total DNA content of hybrid cell lines, necessitating the screening of large numbers of recombinant clones. Preferably, the average insert length should be large to identify most of the human inserts through the presence of human specific interspersed repeats. The handling of a few hundred human clones requires rapid and large scale DNA isolation and hybridization procedures.

In our hands, the lambdaZAP cloning system fulfills these criteria satisfactorily. The *in vitro* packaging of lambda constructs provides for a cloning efficiency which exceeds that of plasmid transformation by a factor of 100. Making use of *in vivo* excision, recombinants can be converted directly into plasmid clones, thus obviating labour intensive subcloning. Also, there is no bias for small inserts as is often observed in plasmid cloning.

Owing to the limited insert size of the lambdaZAP constructs (10 kb; ref. 40), the percentage of clones with human inserts that can be detected through hybridization with total human DNA is lower compared to vectors that allow for larger inserts (lambda-replacement or cosmid vectors). Moreover, the relatively small average insert size renders isolation of low or single copy sequences from human clones difficult. Still, these disadvantages are compensated by the speed and convenience of the lambdaZAP system.

We screened 100 phage containing agar plates for the isolation of 232 human recombinants, to minimize cross-contamination between neighbouring plaques. It is possible to reduce the number of plates to be tested by an alternative screening protocol. If lambdaZAP recombinants are first amplified, pooled, and converted into pBluescript constructs, human plasmid clones can be identified by screening of bacterial colonies. These can be plated at a higher density (approximately 1500/130 mm plate versus 300/130 mm plate for phage plaques), without a higher risk of cross-contamination.

The human X-chromosome comprises approximately 2% of the total genome of the 578 cell line, as estimated using dosage analysis of several X-probes hybridized to Southern blots containing 578 and human male DNA (results not shown). In this study we observed that 0.8% (Table 1) of all clones carried a human insert. As discussed above, we may have missed a considerable percentage of human clones due to the small average insert size (6−7 kb). Also, we performed hybridization of human DNA sequences to phage DNA under stringent hybridization conditions, and thereby might have overlooked some of the human inserts.

5.6% (7/124) of the human clones that showed interpretable hybridization signals could be assigned to the 625 kb *SfiI* fragment. Assuming a total length of the X-chromosome of 200 million bp, this relates to an enrichment of 18-fold, which is comparable to the 37-fold found in a study in which an 840 kb *SfiI* fragment was enriched using preparative PFGE (39).

Three clones isolated from the 625 kb *SfiI* fragment were studied in detail. pZ521 was localised to all three 'classical TCD' deletions used in this study, and was shown to map to the immediate vicinity of a previously isolated jumpclone pJ15 (see Fig. 1 in ref. 15). pZ172 detects a RFLP centromeric to the TCD gene, at an estimated distance of 150−500 kb. pZ11 maps telomeric to the 3.5 deletion, and shows two RFLPs. These three RFLPs should become valuable markers for the diagnosis of the disease in families.

In previous studies, we have characterized a DNA segment of approximately 230 kb, which carries part of the TCD gene and is completely contained within a *SfiI/SalI* fragment of 350 kb (15). Since pZ11 maps to the same 350 kb fragment, its distance from pJ60, the most telomeric jumpclone isolated sofar, must be less than 120 kb. Recent experiments have indicated that pZ11 detects two additional deletions in patients with TCD. These deletions do not overlap deletion 3.5, nor probe pJ60 (manuscript in preparation). This indicates, that the TCD gene may well extend to, and include the pZ11 locus. Therefore the ongoing molecular characterization of this region and the search for evolutionary conserved and expressed sequences in the vicinity of pZ11 should contribute to the isolation of this gene.

ACKNOWLEDGMENTS

The authors are indebted to F.Brunsmann and Dr.I.H.Pawlowitzki for providing blood from patients with TCD. We thank F.Oerlemans for technical assistance in cell culturing. This work was supported by Grant 900-504-064 of the Durch 'Stichting voor Medisch Onderzoek en Gezondheidszorg' (Medigon NWO).

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Chapter 7

Cloning of the breakpoints of a deletion associated with choroideremia

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Hum. Genet. 86:61-64,1990

Cloning of the breakpoints of a deletion associated with choroideremia*

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Received April 2, 1990 / Revised June 1, 1990

Summary. In order to characterize a previously described submicroscopic deletion encompassing (part of) the choroideremia (tapetochoroidal dystrophy: TCD) gene, we have cloned a 10.5-kb *Eco*RI fragment from the patient's DNA; this fragment carries the junction between both deletion endpoints ("junction fragment"). The distal portion of this fragment defines a new marker within, or just distal to, the TCD gene. This marker has been employed to confirm the diagnosis in several affected family members, and to rule out carriership in a female at risk with conspicuous clinical signs.

Introduction

Tapetochoroidal dystrophy (TCD) is an X-linked disorder of the retinal pigment epithelium (RPE), choroid, and retina; it causes progressive nightblindness and visual field constriction in affected males (Goedbloed 1942; Waardenburg 1942; McCulloch and McCulloch 1948). Female carriers generally show characteristic pigment mottling in the midperiphery or posterior pole; this closely resembles fundus anomalies observed in affected young males (Pameijer et al. 1960; Krill 1967). In most cases, the fundus signs in TCD carriers are stationary and cause no visual field defects.

The gene locus for TCD has been assigned to Xq21 by analysis of deletions in male TCD patients with complex phenotypes (Hodgson et al. 1987; Nussbaum et al. 1987; Schwartz et al. 1988; Cremers et al. 1988, 1989a). Molecular characterization of the Xq21 region in patients with classical TCD revealed microdeletions that spanned the DXS165 locus, but none of the other loci tested (Cremers et al. 1987). Cloning of several new markers in the vicinity of the DXS165 locus by use of chromosomal walking and jumping techniques enabled us to define the size of a DNA segment that is spanned by four microdeletions, and to characterize the X-chromosomal breakpoint of a de novo X;13 translocation that was identified in a female with TCD (Cremers et al. 1989b).

Here we report on the cloning and characterization of a DNA fragment that carries the junction between the endpoints of a previously described microdeletion that encompasses (part of) the TCD gene (Cremers et al. 1987, 1989b). The junction fragment was employed to perform reliable carrier detection in the patient's family.

* This work was presented in part at the 5th International Retinitis Pigmentosa Congress, Melbourne 1988

Offprint requests to F. P. M. Cremers

Materials and methods

TCD patients

The TCD family shown in Fig. 3 forms part of a five generation family previously described in detail by Diekstall and Demeler (1988). All members except individuals 7 2 and 7.12 were examined by funduscopy and functional ophthalmologic methods. TCD was diagnosed in three males (7 6, 7 7, 7 11). Two obligate carrier females (7.1, 7.4) showed the expected pigmentation alterations in the midperiphery of the fundus. In a possible female carrier (7 8), fundus characteristics were inconclusive (Diekstall and Demeler 1986, 1988).

Cloning of the 7.6 deletion-junction fragment

DNA from patient 7.6 (100 µg) was digested with *Eco*RI; fragments were ethanol precipitated, and resolved electrophoretically on a 0.7% low-gelling-temperature (LGT) agarose gel. Flanking lanes containing size markers were subsequently cut off and stained with ethidium. The unstained central part of the gel was cut into 2 mm-wide strips perpendicular to the running direction. A small piece of each strip, corresponding to one lane of DNA, was melted for 5 min at 95°C, and brought to 0.3–0.5 *M* NaOH. The samples were spotted onto a Hybond-N membrane by means of a slot-blot apparatus that had been prewarmed to 37°C. DNA (4 µg) from a male, female, and a cell line containing four X-chromosomes (LCL127) were used as controls. The membrane was neutralized by washing in 0.5 *M* TRIS-HCl pH 7.5, 1.5 *M* NaCl, 1 m*M* EDTA for 10 min, air-dried, and exposed to UV-light (305 nm) for 3 min.

The membrane was hybridized to probe p1bD5-II, a single copy sequence located just centromeric to p1bD5, and washed as recommended by the manufacturer. A positive hybridization signal at 10.5 kb was observed Next, the LGT-agarose strip containing DNA from the 10.5 kb *Eco*RI fragment was rinsed three times

in 10 m*M* TRIS-HCl pH 7.5, 100 m*M* NaCl, 1 m*M* EDTA, melted at 65°C for 10 min, and incubated overnight with 50 U agarase/ml (Calbiochem) at 37°C. DNA (2 µg) was recovered by sequential phenol and chloroform extraction, and by ethanol precipitation in the presence of 0.3 *M* NaAc, pH 6. A portion (120 ng) of the 10.5 kb *Eco*RI fraction was ligated to 500 ng of *Eco*RI and *Sac*I doubly cleaved lambda DASH DNA (Stratagene, La Jolla, Calif). The ligation mixture (310 ng) was packaged in vitro using Promega packaging extract. After plating on *Escherichia coli* strain P2392, 95000 clones were obtained. Identification of p1bD5-II positive plaques and subcloning of DNA fragments into plasmid vectors pGEM3 (Promega) or pBluescript (Stratagene) were performed according to published procedures (Maniatis et al. 1982). The nucleotide sequences of DNA fragments p1bD5-I and pJ7.6 were determined in one strand employing the supercoil sequencing method, essentially as given by Chen and Seeburg (1985), and Hattori and Sakaki (1986).

Southern blot analysis

Chromosomal DNA was isolated as described by Aldridge et al. (1984), with minor modifications. DNA (10 µg) was digested with the appropriate restriction enzyme, fragments were resolved by agarose gel electrophoresis and blotted onto Gene Screen Plus (NEN) membranes as described (Cremers et al. 1989a). All probes employed have been described elsewhere (Cremers et al. 1989a, b). Insert DNAs were electrophoretically separated from the plasmid vector and isolated in LGT-agarose (Biorad). Radioactive probes were prepared by random oligonucleotide priming (Feinberg and Vogelstein 1983, 1984). Prior to hybridization, probes containing repetitive sequences (p1bD5, p1bD5-I, p1bD5-II, pJ60, pJ7.6) were preassociated with sonicated human DNA as described by Litt and White (1985). Details of (pre)hybridization and washing of filters were as published (Church and Gilbert 1984).

Field inversion gel electrophoresis (FIGE) analysis

Methods for the preparation of high molecular weight LCL127 (48,XXXX) DNA in agarose blocks, restriction enzyme digestion, FIGE, and blotting, have been described in detail elsewhere (Cremers et al. 1989a).

Results

Cloning of the 7.6 deletion junction fragment

In a previous study, we isolated a cosmid spanning the DXS165 locus (c237; Cremers et al. 1989b). Single- or low-copy fragments were subcloned, gel-purified, and used as probes. The proximal endpoint of the deletion in patient 7.6 was mapped between p1bD5 and a more centromeric subclone of c237, p1bD5-IV (Cremers et al. 1989b). With probe p1bD5-I or -II, Southern analysis of *Eco*RI digested DNA from patient 7.6 and a male control revealed a normal band of 7.8 kb in the control male, and an altered (junction) fragment of 10.5 kb in patient 7.6 (Fig. 1b). Probe p1bD5-I detects a 0.9-kb *Hin*dIII band in DNA of patient 7.6, whereas in healthy controls, a 0.5-kb band is seen (results not shown). This indicates that the proximal endpoint of this deletion must be located within the p1bD5-I sequence (Fig. 1a).

The 10.5-kb *Eco*RI fragment was gel-purified and cloned into lambda DASH phage vector DNA. Probes p1bD5-I and -II detected 4 clones out of 95000 recombinants, one of which was analyzed in detail. The restriction pattern of this clone (λ J7.6; Fig. 1a) agreed with that expected for the rearranged chromosome segment. The authenticity of the junction clone was determined by Southern analysis of genomic DNAs from the deletion patients 3.5, 7.6, 25.6, and a control male, and by detailed analysis of λ J7.6 plasmid-borne subclones. The 6.0-kb *Hin*dIII-*Eco*RI fragment originating from the telomeric part of λ J7.6 (Fig. 1a) predominantly contains

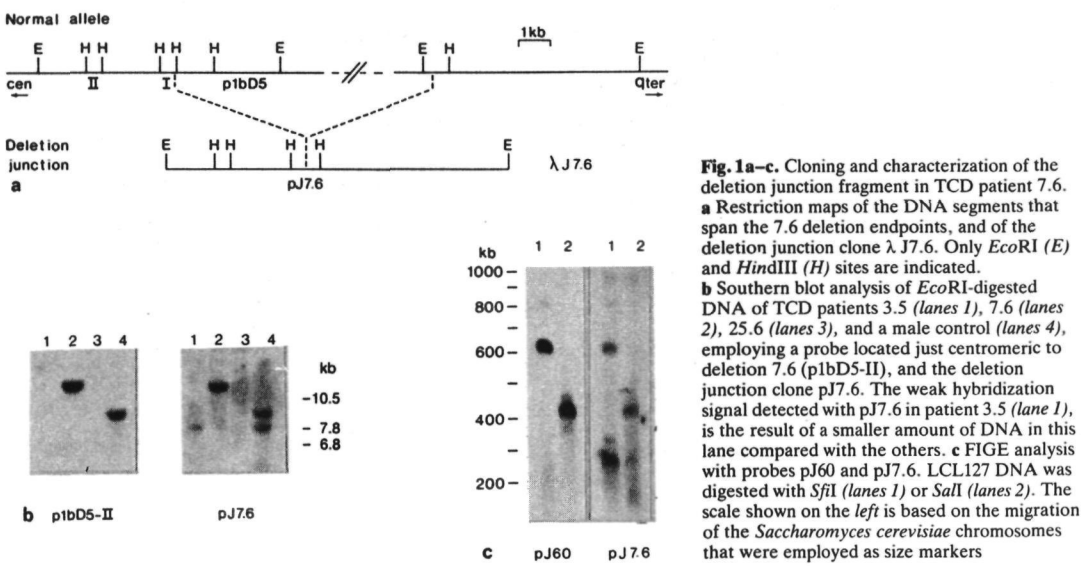

Fig. 1a–c. Cloning and characterization of the deletion junction fragment in TCD patient 7.6. **a** Restriction maps of the DNA segments that span the 7.6 deletion endpoints, and of the deletion junction clone λ J7.6. Only *Eco*RI *(E)* and *Hin*dIII *(H)* sites are indicated. **b** Southern blot analysis of *Eco*RI-digested DNA of TCD patients 3.5 *(lanes 1)*, 7.6 *(lanes 2)*, 25.6 *(lanes 3)*, and a male control *(lanes 4)*, employing a probe located just centromeric to deletion 7.6 (p1bD5-II), and the deletion junction clone pJ7.6. The weak hybridization signal detected with pJ7.6 in patient 3.5 *(lane 1)*, is the result of a smaller amount of DNA in this lane compared with the others. **c** FIGE analysis with probes pJ60 and pJ7.6. LCL127 DNA was digested with *Sfi*I *(lanes 1)* or *Sal*I *(lanes 2)*. The scale shown on the *left* is based on the migration of the *Saccharomyces cerevisiae* chromosomes that were employed as size markers

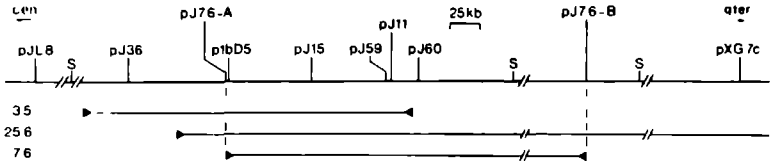

Fig. 2. Deletion map of the TCD locus at Xq21.2. Deleted regions are depicted between *arrowheads*. The centromeric endpoint of the deletion in patient 3 5 is not accurately mapped as indicated by a *dashed line*. Three *SfiI (S)* sites are indicated to underscore the finding that pJ7 6-B is located on a 230-kb *SfiI* fragment that does not span any of the other DNA probes shown here

moderate or highly repetitive sequences (not shown). The actual deletion-breakpoint containing subclone pJ7.6 (0.9-kb *Hin*dIII) proved to be a low-copy probe, and subsequently was used in Southern analysis. In addition to the bands detected by p1bD5-II, pJ7.6 hybridizes to a 6.8-kb *Eco*RI fragment in patient 3.5 and control male (Fig. 1b). This *Eco*RI band is detected by the part of clone pJ7.6 (pJ7.6-B) that is located distal to the breakpoint junction, telomeric to the 3.5 deletion, and completely contained within the 25.6 deletion (Fig. 2). Nucleotide sequencing of clones pJ7.6 and p1bD5-I revealed that the junction fragment contains 466 bp of the region flanking the centromeric deletion endpoint, and approximately 430 bp flanking the telomeric deletion endpoint.

FIGE analysis

To determine the physical location of pJ7.6-B, we hybridized pJ7.6 to a FIGE blot containing *Sfi*I- and *Sal*I-digested control DNA. As expected, because approximately half of the clone (pJ7.6-A) originates from the DXS165 locus, pJ7.6 detects the *Sfi*I and *Sal*I bands of 625 kb and 400 kb, reported earlier (Cremers et al. 1989a). In addition, pJ7.6 detects a 230-kb *Sfi*I band, and two faint *Sal*I bands of 230 kb and 125 kb (Fig. 1c). From these data, combined with the data from deletion analysis, we conclude that pJ7.6-B is located at an unknown distance telomeric to pJ60, but centromeric to pXG7c (Fig. 2).

Carrier detection in family 7

Hybridization of probe p1bD5 to a blot containing *Eco*RI-digested DNA from members of family 7 (see Fig. 3), identifies a deletion in the affected males 7.6, 7.7, and 7.11, as expected. Dosage analysis carried out in order to determine TCD carriership in individuals at risk was hampered by different factors, such as insufficient availability of DNAs, blotting conditions, and nonspecific degradation of DNA. With the pJ7.6 probe, unambiguous proof could be obtained for the carrier status of females 7.1 and 7.4, as they display the junction 10.5-kb *Eco*RI band next to the normal 6.8-kb and 7.8-kb *Eco*RI fragments (Fig. 3). Two individuals at risk for carrying the TCD deletion, namely female 7.8 and male 7.5, showed only the normal 6.8-kb and 7.8-kb *Eco*RI bands.

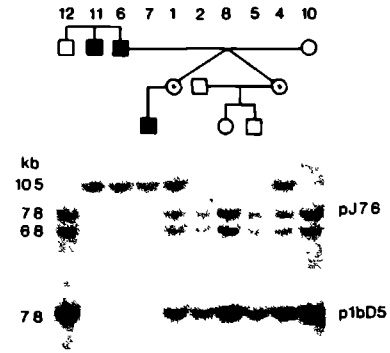

Fig. 3. Southern blot analysis of *Eco*RI-digested DNA of individuals from family 7 with probes pJ7.6 and p1bD5

Discussion

As a prerequisite for cloning of the TCD gene, we isolated several clones from the relevant DNA region by chromosomal jumping (Cremers et al. 1989b). In addition, we have obtained markers from a library highly enriched for sequences from the TCD locus by preparative FIGE (Pol et al. 1990). As a third approach, described here, we have cloned the junction fragment (pJ7.6) of a submicroscopic deletion associated with TCD. FIGE analysis of control DNA indicated that the junction clone detects two different *Sfi*I fragments. Therefore, the corresponding deletion is not confined to the 625-kb *Sfi*I fragment, which was previously shown to carry part of the TCD gene, and we have to conclude that deletion 7.6 extends further distally, encompassing at least one, and possibly several, other *Sfi*I fragment(s).

Cloning of the proximal deletion breakpoint and the corresponding junction fragment enabled us to provide a reliable diagnostic test for relatives of patient 7.6. The presence of a deletion-specific junction fragment confirmed the diagnosis of TCD in three affected males and the obligate carrier state of two females. The absence of the junction fragment in the DNA of a boy (7.5) and a girl (7.8), both at risk of inheriting the deletion from their carrier mother, ruled out TCD and carriership of TCD in these individuals, respectively. It is noteworthy that at the age of 8 years, the fundi of individual 7.8 showed some small midperipheral pigmentations that were taken as being indicative of TCD carriership. Therefore, our finding illustrates that, whenever possible, carrier diagnosis in TCD should be corroborated by DNA analysis. Recent data from our laboratory indicate that

similar deletions encompassing part of the TCD gene can be detected in at least 10% of the patients (unpublished results). In most other families, unambiguous diagnostic results can be obtained with closely linked probes (Pol et al. 1990).

As depicted in Fig. 2, pJ7.6-B is located between the most telomeric jump clone (pJ60) and the anonymous probe pXG7c (DXS95), and is therefore useful as a new molecular entry site for the chromosomal segment that carries the TCD locus. Indeed, employing pJ7.6 as a probe, we have recently detected two microdeletions associated with TCD, one of which had not yet been identified with any of the anonymous markers and jump clones available from the Xq21.2 region (Cremers et al., 1990). Characterization of these small deletions will further assist in defining the genomic size of this locus and eventually help to clone the TCD gene itself.

Acknowledgements. This work was supported by Grant 900-504-064 of the Dutch "Stichting voor Medisch Onderzoek en Gezondheidszorg" (Medigon NWO).

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Chapter 8

Deletions in patients with classical choroideremia vary in size from 45 kb to several megabases

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Am. J. Hum. Genet. 47:622-628,1990

Deletions in Patients with Classical Choroideremia Vary in Size from 45 kb to Several Megabases

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Summary

Making use of the p1bD5 probe (DXS165), we have isolated several markers from the choroideremia locus by chromosomal jumping, preparative field-inversion gel electrophoresis, and cloning of a deletion junction fragment. With these clones we were able to identify and characterize eight deletions in 69 choroideremia patients investigated. The deletions are heterogeneous, in both size and location. The smallest deletion (patient LGL1134) comprises approximately 45 kb of DNA, whereas the largest ones (patients 25.6 and LGL2905) span a DNA segment of at least 5 megabases, which is comparable in size to the smallest deletion detected in a TCD patient (patient XL45) showing a complex phenotype. The TCD deletions encompass variable parts of a 150–200-kb DNA segment that is flanked by p1bD5 (DXS165) at the centromeric side and by pZ11 at the telomeric side. The deletions in patients 33.1, LGL1101, and LGL1134 do not span a translocation breakpoint which was previously mapped on the X chromosome of a female with TCD. The clones isolated from the TCD locus are valuable diagnostic markers for deletion analysis of patients or carrier females. In addition, they should be useful for the isolation of expressed sequences that are part of the TCD gene.

Introduction

Cytogenetically visible deletions in the X chromosome of males are almost invariably associated with contiguous gene syndromes and are predominantly located in Giemsa dark-staining bands. The absence of large DNA segments in Xp22.3 and Xp21 results in complex disorders, of which ichthyosis and Duchenne muscular dystrophy, respectively, are the most prominent features (Curry et al. 1984; Francke et al. 1985; Wieringa et al. 1985; Saito et al. 1986; Pillers et al. 1988). Deletions of various parts of Xq21 are associated with choroideremia (tapetochoroidal dystrophy [TCD]), mental retardation (MR), deafness, or other symptoms, including cleft lip and palate (CLP) (Rosenberg et al. 1986; Schwartz et al. 1986, 1988; Hodgson et al. 1987; Nussbaum et al. 1987; Cremers et al. 1988, 1989*a*; Merry et al. 1989). Independent evidence for the TCD gene being located at Xq21 has been obtained by linkage studies (Lewis et al. 1985; Nussbaum et al. 1985; Jay et al. 1986; Schwartz et al. 1986; Lesko et al. 1987; MacDonald et al. 1987; Sankila et al. 1987, 1989).

Deletion mapping of five TCD patients showing a complex phenotype enabled us to subdivide the Xq21 band into seven intervals (Cremers et al. 1989*a*). The TCD gene could be positioned in interval 3, which is defined by the loci DXS233, DXS165, and DXS95. Furthermore, in two females with TCD, this condition was associated with different chromosomal translocations, both involving Xq21.2 (Kaplan et al. 1989; Siu et al. 1990).

The position of the TCD gene has been determined

Received February 15, 1990; revision received April 26, 1990.

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0002-9297/90/4704-0006$02 00

more precisely by the molecular identification of microdeletions that span the DXS165 locus (probe p1bD5, Cremers et al. 1987*a*) in the DNA of patients with classical TCD. Chromosomal walking and jumping from the DXS165 locus enabled us to characterize four of these TCD deletions, as well as the Xq21.2 breakpoint in the DNA of a TCD female with a de novo X;13 translocation (Cremers et al. 1989*b*). The DNA region containing the putative TCD gene was saturated with additional clones by the construction of a library enriched for sequences from a 625-kb *Sfi*I fragment (van de Pol et al. 1990). From this library, several informative clones were obtained, one of which was used in the present study. Also, we have cloned the endpoints of a TCD deletion (Cremers et al., in press), which provided us with an additional marker for the identification and characterization of deletions in patients with TCD.

Material and Methods

TCD Patients

A total of 69 male patients showing the classical TCD phenotype were ascertained by F.B., B.J., A.J.L.G.P., E.-M.S., and M.S. The diagnosis of TCD was inferred from the characteristic history and fundus appearance (Sorsby et al. 1952; Kurstjens 1965; Kärnä 1986), consisting of peripheral pigmentary retinopathy with areas of pigmented epithelial and choroidal atrophy. Retinal vessels are unremarkable even at advanced stages of the disease. The clinical picture of patients 3.5 and 7.6 has been described by Hammerstein and Böhm (1985) and Diekstall and Demeler (1988), respectively. The TCD patients belong to various Caucasian subpopulations, including the Finnish (patients LGL1101, LGL1134, and LGL2905), the German (patients 3.5, 7.6, 25.6, and 33.1), and the British (patient C3).

A female TCD patient who showed mild TCD and infertility secondary to premature ovarian failure was karyotyped as 46,X,t(X;13) (q21.2;p12) by using trypsin-Giemsa staining. Somatic cell hybrids formed between skin fibroblasts from this patient and mouse A9 fibroblasts yielded a mouse-human hybrid (CIII-1), which contained the derivative der(13) chromosome but not the reciprocal der(X) chromosome (Siu et al. 1990).

DNA Markers

The anonymous DNA probes from the Xq21 region (fig. 1), have been described elsewhere (Goodfellow et al. 1985, Cremers et al. 1987*b*, 1988; Nussbaum et al. 1987). Probe pXG8b was provided by P. Szabo; pF1 and pF8 were gifts from T. A. Kruse (Aarhus). The jump clones pJ11, pJ15, pJ36, pJ59, and pJ60 have been described elsewhere (Cremers et al 1989*b*).

pZ11 was isolated from a library enriched for sequences from a 625-kb *Sfi*I fragment, by using preparative field-inversion gel electrophoresis (FIGE). pZ11 contains two *Eco*RI fragments (1.2 and 4.2 kb), which are located next to each other in the X chromosome (van de Pol et al. 1990). The 1.2-kb fragment (pZ11a) contains no repetitive sequences and is used in the present study for mapping purposes. The 4.2-kb fragment (pZ11b) contains highly repetitive sequences (F. P. M. Cremers, unpublished results).

pJ7.6 is a junction clone (0.9-kb *Hin*dIII) which contains sequences from the endpoints of deletion 7.6 (Cremers et al., in press). This clone contains some moderately repetitive sequences requiring preassociation with sonicated human DNA before being used as a probe (see below).

Southern Blot Analysis

Chromosomal DNA was isolated according to a method described by Aldridge et al. (1984), with minor modifications. DNA (10 μg) was digested with the appropriate restriction enzyme, and fragments were resolved by agarose-gel electrophoresis and blotted onto GeneScreenPlus (New England Nuclear) membranes (Cremers et al. 1989*a*). Insert DNAs were electrophoretically separated from the plasmid vector and were isolated in LGT-agarose (Biolab). Radioactive probes were prepared by random oligonucleotide priming (Feinberg and Vogelstein 1983, 1984). Prior to hybridization, probes containing repetitive sequences (i.e., probes pX65H7, pX104f, pF1, pF8, pJ36, pJ60, and pJ7.6) were preassociated with sonicated human DNA (Litt and White 1985). Conditions for (pre)hybridization and washing of filters have been published elsewhere (Church and Gilbert 1984).

Pulsed-Field Gel Electrophoresis (PFGE) Analysis

Methods for the preparation of high-molecular-weight DNA in agarose blocks and restriction-enzyme digestion have been described elsewhere (Cremers et al. 1989*a*). PFGE was carried out using a contour-clamped homogeneous electric field apparatus according to the specifications given by Chu et al. (1986). White blood cells from TCD patients LGL526, LGL1101, LGL1134, LGL1307, and LGL2905 and from a control female were embedded in LGT agarose (van Ommen and Verkerk 1986). Agarose-immobilized DNAs were digested with *Sfi*I, as recommended by the manufac-

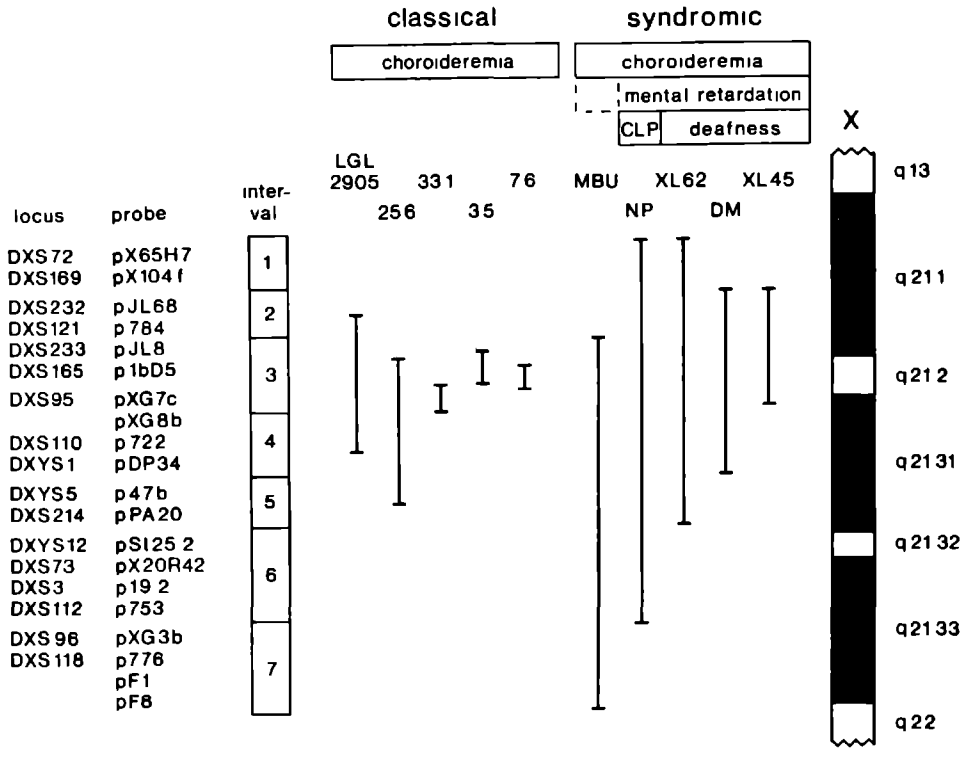

Figure 1 Deletion map of the Xq21 band, with the anonymous DNA probes employed indicated at the left. The ordering of probes in intervals is based on the molecular analysis of syndromic TCD patients, who are depicted at the right side for comparison (see Cremers et al. 1989*a*)

turer. Blocks were subsequently transferred to slots of a 1% (w/v) agarose gel. Electrophoresis was performed in 0.25 × TBE (Maniatis et al. 1982) for 72 h at 14°C and 120 V. The switching interval was kept constant at 120 s for the forward phase and 40 s for the reverse phase. DNA transfer and hybridization methods were the same as previously described for FIGE analysis (Cremers et al. 1989*a*).

Results

Using the anonymous probes depicted in figure 1, we were able to detect a total of five deletions in 69 patients with classical TCD. The deletions found in patients 3.5 and 7.6 are confined to the p1bD5 (DXS165) marker (Cremers et al. 1987*a*). The deletion present in the DNA of patient 33.1 was not detected with p1bD5 but was detected with pXG7c (DXS95), which is also located in interval 3, and is situated telomeric to p1bD5. Patients 25.6 and LGL2905 both carry large interstitial deletions that encompass six anonymous markers. The proximal endpoint of deletion LGL2905 is located in interval 2 between pJL68 (DXS232) and p784 (DXS121). Therefore this breakpoint maps to the 400-kb *Sfi*I fragment which contains both markers (Cremers et al. 1989*a*, Merry et al. 1989). The distal deletion endpoint is located in interval 4, thereby allowing assignment of pDP34 (DXYS1) distal to pXG8b and p722 (DXS110). The proximal deletion endpoint in patient 25.6 is located just centromeric to p1bD5; the telomeric

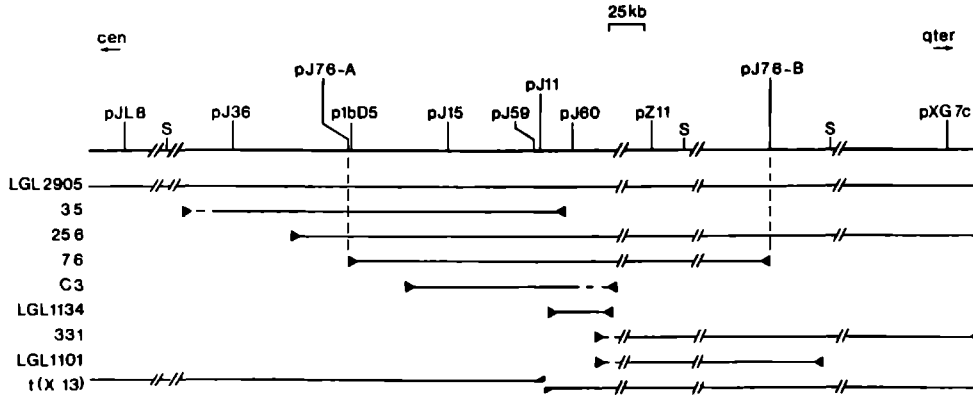

Figure 2 Deletion map of the TCD locus at Xq21 2 Deleted regions are depicted between arrowheads, the location of the X chromosomal breakpoint of a X,13 translocation in a female with choroideremia is shown at the bottom The centromeric endpoints of the deletions in patients 3 5, 33 1, and LGL1101, as well as the telomeric endpoint of deletion C3, are not accurately mapped, as indicated with dashed lines Clones pJ7 6 A and pZ11 are positioned on the 625 kb *Sfi*I fragment which encompasses p1bD5 and the jump clones indicated pJ7 6 B is located on a 250-kb *Sfi*I fragment (van de Pol et al 1990, Cremers et al , in press)

endpoint maps to interval 5 (Cremers et al. 1989*b*). Deletions 25.6 and LGL2905 overlap to a large extent with the complex TCD deletions XL45 and DM (fig 1).

Physical Fine Mapping of Deletions in Classical TCD Patients

Employing all DNA markers indicated in figure 2, we were able to detect eight deletions in 69 TCD patients investigated. The jump clones pJ11, pJ15, pJ36, pJ59, and pJ60 detect six deletions, four of which (3 5, 7 6, 25 6, and LGL1134) have been reported elsewhere (Cremers et al. 1989*b*). Deletion LGL2905 encompasses the entire region depicted in figure 2 (see above; fig. 1). Deletion C3 spans the markers pJ15, pJ59, pJ11, and pJ60.

Clones pZ11 and pJ7.6 have been obtained by employing alternative cloning strategies (van de Pol et al. 1990; Cremers et al., in press). pZ11 is spanned by the deletions 7.6, 25.6, 33.1, LGL1101, and LGL2905 and is located telomeric to the deletions detected in the DNA of patients 3 5, LGL1134, and C3. Southern blotting results for patients LGL1101, LGL1134, and LGL2905 are presented in figure 3. Except for deletion 7.6, pJ7 6-B yields the same hybridization results as does pZ11 (data not shown).

The translocation breakpoint present in the X chromosome of a female showing TCD (46,X,t[X;13] [q21.2;p12]) is located within 2 kb telomeric to pJ11 but just centromeric to deletion LGL1134, as indicated in figure 2. For two patients, C3 and LGL1134, PFGE analysis can allow for an accurate estimation of deletion sizes, since both are completely contained within the 625-kb *Sfi*I fragment. Deletion C3 has not been investigated; the analysis of deletion LGL1134 will be described below.

PFGE Analysis of Deletions

High-molecular-weight DNA from several TCD patients and from a control female was digested with *Sfi*I, resolved by PFGE, and hybridized to probe p1bD5. Compared with controls, p1bD5 detects altered DNA fragments in patients LGL1101 and LGL1134 (see fig. 4, lanes 4 and 6). The deletion found in LGL2905 (see fig. 4, lane 2) spans all loci depicted in figure 2. Patient LGL1101 shows a band of 575 kb, which does not give a clue regarding deletion length, as this deletion extends beyond the 625-kb *Sfi*I fragment (fig. 2). The 580-kb *Sfi*I band observed in patient LGL1134 directly relates to a deletion size of 45 kb, as this deletion is confined to the 625-kb *Sfi*I fragment.

Discussion

TCD is an X-linked chorioretinal dystrophy in which affected males suffer from progressive night blindness and visual field constriction, leading to blindness by

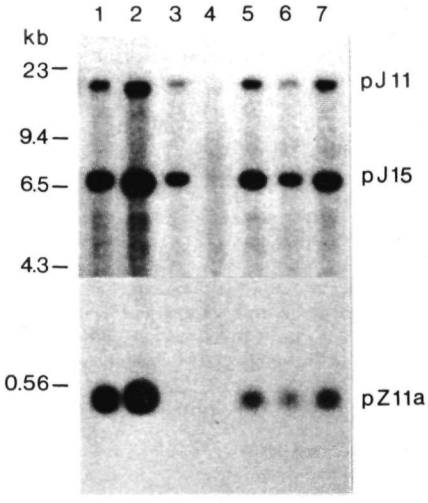

Figure 3 Southern blot analysis of *Taq*I-digested DNA from control female (lanes 1 and 7) and TCD patients LGL1134 (lane 2), LGL1101 (lane 3), LGL2905 (lane 4), LGL526 (lane 5), and LGL1307 (lane 6), with the probes indicated at the right. The upper part of the blot was exposed for a longer time to enhance the signals for pJ11 and pJ15 compared with that for pZ11a.

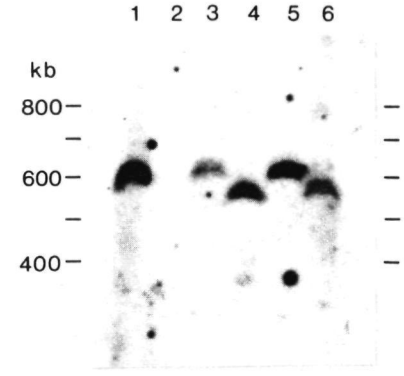

Figure 4 PFGE analysis with probe p1bD5 of *Sfi*I-digested high-molecular-weight DNA from a control female (lane 1) and TCD patients LGL2905 (lane 2), LGL526 (lane 3), LGL1101 (lane 4), LGL1307 (lane 5), and LGL1134 (lane 6). The scale shown on the left is based on the migration of the *Saccharomyces cerevisiae* chromosomes which were employed as size markers.

the third to fourth decade of life (Goedbloed 1942; Waardenburg 1942; McCulloch and McCulloch 1948; Sorsby et al. 1952). Female carriers, although normally asymptomatic, can be detected by characteristic patchy changes in the retinal pigment epithelium that reflect random X inactivation.

In previous studies, we and others have shown that TCD patients showing additional disorders carry deletions that vary considerably in size (Hodgson et al. 1987; Nussbaum et al. 1987; Cremers et al. 1988, 1989*a*; Schwartz et al. 1988; Merry et al. 1989). The smallest deletion associated with a complex phenotype (patient XL45) was estimated to span approximately 5,300 kb, as determined by flow karyotype analysis (Merry et al. 1989). This deletion could not be detected with certainty by high-resolution chromosome analysis but was identified with anonymous DNA markers from Xq21 (Nussbaum et al. 1987). The other four TCD patients with complex syndromes showed microscopically visible deletions in the Xq21 band. On the basis of the size estimate for the deletion in the X chromosome of patient XL62 (12,000 kb; Merry et al. 1989), we assume that the deletions found in patients NP and MBU encompass approximately 15 megabases.

In an earlier report, we described the identification of deletions in TCD patients 3.5 and 7.6. These deletions were presumed to be relatively small, because they only spanned the DXS165 locus, and none of the other anonymous markers from Xq21 tested. The surprising finding in the present study is that patients with classical TCD show deletions that vary in size from approximately 45 kb (patient LGL1134) to several megabases (patients 25.6 and LGL2905). Taken together, the deletions in the latter two patients span part of interval 2, intervals 3 and 4, and part of interval 5. Apart from a small proximal fragment, this DNA segment overlaps almost entirely the previously analyzed deletions of patients DM and XL45 (fig. 1), who show not only TCD but also MR and congenital sensorineural deafness. Therefore, genes involved in MR and deafness may be located in the proximal part of interval 2, a DNA segment defined by the DXS232 locus. Recently, another probe mapping to interval 2, pHU16 (DXS26), was localized centromeric to the LGL2905 deletion (Sankila et al. 1990) and should be of use for further fine mapping of the loci involved in MR and in congenital sensorineural deafness. Patient NP does not show deafness despite the fact that his deletion overlaps the entire interval 2, indicating that the absence of this interval predisposes for—but does not necessarily cause—hearing impairment.

To define the subchromosomal region carrying the TCD gene, we have isolated DNA clones by making

use of (1) chromosomal walking and jumping from the DXS165 locus (Cremers et al. 1989*b*), (2) construction, by preparative FIGE (van de Pol et al. 1990), of a library enriched for sequences from a 625-kb *SfiI* fragment, and (3) forced cloning of a gel-purified deletion junction fragment of the deletion observed in patient 7.6 (Cremers et al., in press). In a total of 69 TCD patients investigated with these probes, we were able to detect and characterize eight deletions, four of which could be identified with probe p1bD5 (DXS165). This finding is remarkable in view of the results of Merry et al. (1990), who did not detect any deletion with probe p1bD5 (DXS165) in 42 unrelated TCD probands.

The deletions found in patients 33.1 and LGL1101 do not overlap the deletion in patient 3.5. Cloning of the LGL1134 deletion junction fragment, by making use of pJ11 or pJ59, will enable us to determine the extent of nonoverlap of several deletions in this region. The location of the X;13 translocation breakpoint just centromeric to the 45-kb LGL1134 deletion allows for an estimate of the minimal size of the TCD gene. On the basis of restriction analysis of the pJ59/pJ11/pJ60 region, it must span at least 35 kb of genomic DNA. Employing the currently available set of DNA sequences from the TCD gene region, a search for evolutionarily conserved and expressed sequences is in progress, which should yield further insight into the molecular organization of this locus.

Acknowledgments

The authors are indebted to Drs. G A Bruns, D Drayna, M H. Hofker, T A Kruse, J. L Mandel, D Page, K Smith, P Szabo, J Weissenbach, and B N. White for supplying DNA probes. We thank W F Flintoff for sharing cell line CIII-1 and F Oerlemans and L van Rossum for technical assistance in cell culturing. This work was supported by the Nederlandse Organisatie voor Wetenschappelijk Onderzoek grant 900-504-064 and by grants from The Sigrid Juselius Foundation, The Academy of Finland, and The Folkhalsan Institute of Genetics.

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Chapter 9

Cloning of a gene that is rearranged in patients with choroideremia

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Nature (London) 347:674-677,1990

Cloning of a gene that is rearranged in patients with choroideremia

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CHOROIDEREMIA (tapetochoroidal dystrophy, TCD), a common form of X-linked blindness[1], is characterized by progressive dystrophy of the choroid, retinal pigment epithelium and retina[2,3]. Previous studies have assigned the TCD gene to a small segment of the Xq21 band[4–6]. By making use of reverse genetics strategies we have isolated eight overlapping complementary DNA clones from the same chromosomal region. The corresponding gene is expressed in retina, choroid and retinal pigment epithelium. The cDNAs encompass an open reading frame of 948 base pairs that is structurally altered in eight TCD patients with deletions, and *in a female patient with a balanced translocation involving* Xq21. These findings provide strong evidence that we have cloned the gene underlying choroideraemia. Elucidation of its function should provide new insights into the molecular mechanisms responsible for this disorder and other hereditary retinopathies.

In previous studies we and others have shown that the TCD gene maps to an interval of the Xq21 band defined by the DNA markers DXS95, DXS165 and DXS233[7,8]. One of these loci,

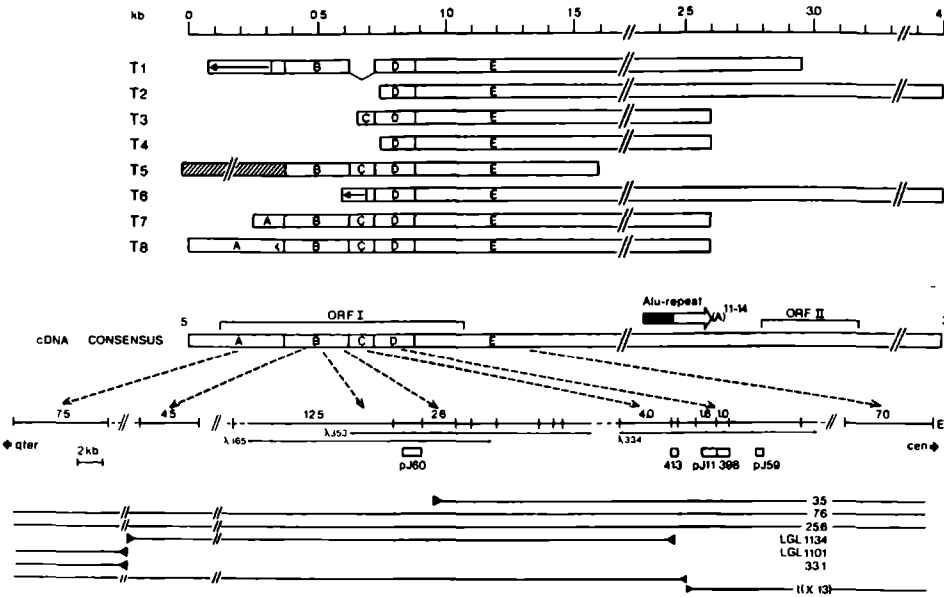

FIG. 1 Overlapping cDNA clones, their alignment with respect to genomic DNA fragments as well as deletion and translocation breakpoints in the Xq21 region Top, the relative positions of the eight cDNAs shown were deduced from restriction enzyme mapping and sequence analysis Genuine exon sequences in cDNA inserts are represented by open boxes A–E denote distinct cDNA segments, each of which may be composed of several exons Vertical bars represent exon–exon boundaries as inferred from comparison with genomic DNA sequences (clones pJ11 and 398 for defining 5' and 3' borders of segment D, respectively) or from comparisons between different cDNAs (T1, T5, T7, and T8 for both borders of segment C and the 3'-border of segment A) The cross-hatched box at the 5' end of T5 probably represents an intron sequence as a perfect splice acceptor splice site sequence[13] was identified directly upstream of segment B in this clone Four clones (T3, T4, T7 and T8) with truncated 3' ends probably originate from oligo(dT) primed reverse transcription starting at a poly(A) stretch located just 3' of an internal Alu repeat, which is indicated by a boxed arrow Clones T1 and T5 probably result from alternatively or aberrantly spliced precursor RNAs Both the segment lacking from T1 (segment C) and the extra stretch identified at the 5' end of T5 (cross-hatched segment) feature genuine splice site signals[13] at their borders Arrows in clones T1 and T6 indicate sequences that are reversed with respect to the sequence determined for the combined cDNAs *These reversed segments contain short inverted repeats at their* ends and probably originate from aberrant processing of stem-loop structures during reverse transcription of the mRNA Bottom, the consensus cDNA as deduced from eight cDNA clones is shown in relation to a partial genomic map of the corresponding chromosome segments Deletions are shown as horizontal bars between triangles The X-chromosomal breakpoint in a female with a balanced t(X,13) translocation[5,15] is indicated The positions of the two longest ORFs in the cDNA are given The partial *Eco*RI map was constructed by Southern analysis of *Eco*RI-digested genomic DNA, and by restriction analysis of three recombinant phages (λ334, λ353 and λ365) The chromosomal origin as well as the telomere–centromere orientation of the cDNA segments A–E is indicated The outermost sections of the cDNA are homologous to *Eco*RI fragments that are still unlinked to the central region of the genomic map

METHODS Genomic DNA phage clones with human inserts were isolated employing jump clones pJ60, pJ11 and pJ59 (see ref 5) using standard cloning methodology Phage 353 and 365 originated from a total human DNA genomic library in phage EMBL3 (courtesy of G Grosveld, Rotterdam). Phage 334 was isolated from a pool of recombinant λ DASH (Stratagene) phage containing 14 5 kb *Hin*dIII inserts enriched by preparative gel electrophoresis (for methods, see ref 9) Plaque purification, DNA isolation, restriction enzyme mapping and subcloning of phage-insert fragments into pGEM 3/4 plasmid vectors (Promega Biotec) was done according to standard recombinant DNA procedures[23] Single-copy *Eco*RI inserts identified in plasmids 398 and 413 were subsequently used as probes on zoo blots (see Fig 3*a*) and for screening a λgt10 human retinal cDNA library[12]. Phage recombinants (10^6) were screened with a mixture of ^{32}P-labelled[24] 398 and 413 insert DNAs using standard procedures[23] Eight positive cDNA clones, T1–T8, were plaque-purified Insert DNA prepared from plate lysates of each of the eight recombinant phage was cleaved with *Eco*RI and subcloned in plasmid pGEM4 Alignment of various cDNAs was based on restriction mapping using restriction enzymes *Acc*I, *Bgl*II, *Eco*RI, *Hin*dIII, *Nco*I and *Pst*I and on sequence analysis determination (see Fig. 2). The region between nucleotide positions 1 and 1 000 shown at the top of the figure and a stretch of ~250 nucleotides at the 3' ends were sequenced in all cDNA clones. Only for clone T6 was the insert completely sequenced

DXS165, is deleted in several patients with classical TCD[4–6]. As a prerequisite for the molecular characterization of the TCD gene region, deletion-breakpoint cloning[9], preparative field inversion gel electrophoresis[10], and walking and jumping techniques[5,11] were used to generate new DNA markers near the DXS165 locus. Using these new markers, a genomic segment of approximately 45 kilobases (kb) which overlaps most of the TCD-associated deletions (Fig. 1) could be cloned. From this segment, 15 single-copy sequences were isolated and screened for evolutionary conservation by hybridization to genomic DNA from various vertebrate species. With two of these DNAs, probes 398 and 413 (Fig. 1), specific hybridization signals were obtained with DNAs from several species including chicken (Fig. 3*a*). On northern blots, probe 398 yielded distinct signals in RNAs from retina and retinal cell lines (not shown). Screening of a human retinal cDNA library[12] resulted in the isolation of eight overlapping cDNA clones (T1–T8), which span a total of 4.5 kb but do not contain the 5′ and 3′ ends of the gene (Fig. 1). Clone T8 extends furthest upstream and contains an open reading frame (ORF) (Fig. 2*a*, ORFI) capable of encoding a polypeptide of 316 amino acids. Nuclease S1 analysis indicated that the insert of this clone is fully protected by messenger RNA from a retinal cell line up to about 55 nucleotides from its 5′ end (Fig. 2*b*). The sequence immediately upstream of position 56 (Fig. 2*a*; underlined) has a conspicuous resemblance to the consensus sequence for 3′ splice sites[13], which may indicate that these nucleotides originate from an intron extending further upstream. At position 61–63, there is a stop codon, and the first ATG codon of the ORF at position 127–129 may function as a translation start site[14].

None of the cDNA clones contains the poly(A) sequences normally indicative of the 3′ end of mRNAs. The complete sequence deduced from the combined cDNAs (not shown) contains a strikingly large 3′ untranslated region of 3.4 kb. In the middle of this region, starting at position 2,800 and adjacent to a sequence that could act as a 3′ splice site, we have found an ORF spanning 395 base pairs (bp), (Fig. 1; ORFII). The functional significance of this sequence remains to be elucidated.

To ascertain the relationship between the cloned cDNA and the TCD gene, genomic DNAs of eight patients with TCD who carried submicroscopic deletions of the Xq21 band[5,6] were re-examined with single-copy subclones of cDNA T1. In all patients the deletions had removed at least part of the long ORF that spans segments A to E of the consensus cDNA (Figs 1 and 3*b*). In addition, we could locate the position of the X-chromosomal breakpoint of a t(X; 13) translocation previously identified in a female with typical signs of TCD[5,15], to intron sequences that separate segments C and D of the cDNA (Fig. 3*c*). In combination, these data provide strong evidence that the cloned cDNA is indeed part of the choroideraemia gene.

RNA analyses have corroborated this conclusion. In choroid/retinal pigment epithelium, retina, two retinal cell lines (HER XC2 and HER RC2; ref. 16), and HeLa cells, all eight cDNA clones hybridize to an mRNA transcript of ~5,400 residues (Fig. 4*a*). Surprisingly, this mRNA is also expressed in Epstein–Barr virus-immortalized B cells, albeit at a significantly lower level. This finding enabled us to study mRNA patterns in patients with deletions of various size and should pave the way for the detection of point mutations in the TCD gene. As shown in Fig. 4*b*, with a probe from the 5′ end of the cDNA (T1E0.5), no hybridization signals were seen on northern blots containing RNAs from lymphoblastoid cell lines of TCD patients 7.6 and 25.6. This is not unexpected as both deletions span the entire gene. In contrast, patient 3.5, who lacks the 3′ end of the gene, shows aberrant transcripts of about 4.5 and 5.5 kb. In this patient, two different novel sites may be used as polyadenylation signals.

Neither the nucleotide nor the predicted amino-acid sequence of the putative gene product revealed any significant homology to genes or protein sequences in the NBRF (December 1989), Swiss-Prot (January 1990) and EMBL (April 1990) databases. The protein carries potential phosphorylation sites for protein kinase C (residues 76, 122, 175, 178, 301) and for casein kinase (residues 13, 129, 178, 195, 239, 301), but no other sites associated with post-translational modification processes. Moreover, no topogenic sequences, no domains with known biological

a

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CGGAGAAGACCAAGGAGGGCGTCCTCTACGTCGGAAGCAAGACCTTGTTCATCAGGATAT   60

TAAGAGATCACATTTTATGAATATTTAAAGACTCAAAAATTAACCCCCAACCTCCAATAT  120
*
ATTGTCATGCATTCAATTGCAATGACATCAGAGACAGCCAGCAGCACCATAGATGGTCTC  180
      M  H  S  I  A  M  T  S  E  T  A  S  S  T  I  D  G  L     18

AAAGCTACCAAAAACTTTCTTCACTGTCTTGGGCGGTATGGCAACACTCCATTTTTGTTT  240
 K  A  T  K  N  F  L  H  C  L  G  R  Y  G  N  T  P  F  L  F    38

CCTTTATATGGCCAAGGAGAACTCCCCCAGTGTTTCTGCAGGATGTGTGCTGTGTTTGGT  300
 P  L  Y  G  Q  G  E  L  P  Q  C  F  C  R  M  C  A  V  F  G    58

GGAATTTATTGTCTTCGCCATTCAGTACAGTGCCTTGTAGTGGACAAAGAATCCAGAAAA  360
 G  I  Y  C  L  R  H  S  V  Q  C  L  V  V  D  K  E  S  R  K    78

TGTAAAGCAATTATAGATCAGTTTGGTCAGAGAATAATCTCTGAGCATTTCCTCGTGGAG  420
 C  K  A  I  I  D  Q  F  G  Q  R  I  I  S  E  H  F  L  V  E    98

GACAGTTACTTTCCTGAGAACATGTGCTCACGTGTGCAATACAGGCAGATCTCCAGGGCA  480
 D  S  Y  F  P  E  N  M  C  S  R  V  Q  Y  R  Q  I  S  R  A   118

GTGCTGATTACAGATAGATCTGTCCTAAAAACAGATTCAGATCAACAGATTTCCATTTTG  540
 V  L  I  T  D  R  S  V  L  K  T  D  S  D  Q  Q  I  S  I  L   138

ACAGTGCCAGCAGAGGAACCAGGAACTTTTGCTGTTCGGGTCATTGAGTTATGTTCTTCA  600
 T  V  P  A  E  E  P  G  T  F  A  V  R  V  I  E  L  C  S  S   158

ACGATGACATGCATGAAAGGCACCTATTTGGTTCATTTGACTTGCACATCTTCTAAAACA  660
 T  M  T  C  M  K  G  T  Y  L  V  H  L  T  C  T  S  S  K  T   178

GCAAGAGAAGATTTAGAATCAGTTGTGCAGAAATTGTTTGTTCCATATACTGAAATGGAG  720
 A  R  E  D  L  E  S  V  V  Q  K  L  F  V  P  Y  T  E  M  E   198

ATAGAAAATGAACAAGTAGAAAAGCCAAGAATTCTGTGGGCTCTTACTTCAATATGAGA  780
 I  E  N  E  Q  V  E  K  P  R  I  L  W  A  L  Y  F  N  M  R   218

GATTCGTCAGACATCAGCAGGAGCTGTTATAATGATTTACCATCCAACGTTTATGTCTGC  840
 D  S  S  D  I  S  R  S  C  Y  N  D  L  P  S  N  V  Y  V  C   238

TCTGGCCCAGATTGTGGTTTAGGAAATGATAATGCAGTCAAACAGGCTGAAACACTTTTC  900
 S  G  P  D  C  G  L  G  N  D  N  A  V  K  Q  A  E  T  L  F   258

CAGGAAATCTGCCCCAATGAAGATTTCTGTCCCCCTCCACCAAATCCTGAAGACATTATC  960
 Q  E  I  C  P  N  E  D  F  C  P  P  P  P  N  P  E  D  I  I   278

CTTGATGGAGACAGTTTACAGCCAGAGGCTTCAGAATCCAGTGCCATACCAGAGGCTAAC 1020
 L  D  G  D  S  L  Q  P  E  A  S  E  S  S  A  I  P  E  A  N   298

TCGGAGACTTTCAAGGAAAGCACAAACCTTGGAAACCTAGAGGAGTCCTCTGAATAATGG 1080
 S  E  T  F  K  E  S  T  N  L  G  N  L  E  E  S  S  E  *      316

ATATACACCAAACTGGATACCCAACTTTGGAAATTCTGACTGGTCTCAGAGTCTACTTGA 1140

TAGAAGGACTGTTTGAGAAATGTTAGAAAGCAGCAGCAATTATAAGGCAAAATAGGTAAT 1200

AGAAATCCAAAAGGGGATTTTCCTTATAGAGGACATTCCAAGAACACACAACACTTATAA 1260

AGCACATTGACTTGCTCATTTTAAATACCAAACTTGTGTGACTAGCAGATGAAAATTATA 1320

AATCAATTGATTCTCAGGAATGTAACTGTGGATATGAAAGTGATCCTATGCATTGTTAAT 1380

AATTCCATGG                                                    1390
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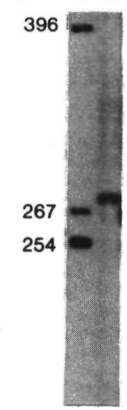
FIG. 2 Structure of the candidate TCD cDNA. *a*, Nucleotide sequence of the region containing the largest ORF and the deduced amino-acid sequence of the corresponding protein. The DNA sequence was derived through combination of several cDNAs and overlaps the ORF indicated in Fig. 1. Upper line, nucleotide sequence; lower line, amino-acid sequence (single-letter code) beginning at the putative methionine initiation codon (see text). A putative 3′ splice site is underlined. Asterisks indicate stop codons. *b*, Nuclease S1 protection assay using a fragment of clone T8. Lane 1, marker DNA is plasmid pGEM4 digested with *Hae*III, dephosphatased, and end-labelled with [γ-^{32}P]ATP. Lane 2, hybridization of the 5′ end of cDNA clone T8 to HER Xc2 mRNA, followed by nuclease S1 digestion, resulted in a protected DNA fragment of ~271 nucleotides.



METHODS. *a*, Inserts of cDNA phage T1–T8 (Fig. 1) were cleaved with restriction enzymes and the resulting fragments were recloned on ligation into the appropriate sites of the polylinker sequence of plasmid pGEM4 (Promega Biotec). Both strands of the relevant plasmid DNAs were sequenced with T7 or SP6 sequencing primers (Promega Biotec) following the procedures of Hattori and Sakaki[25]. *b*, A 330-nucleotide *Eco*RI–*Rsa*I fragment from clone T8 was 5′ end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase, hybridized with 25 μg total HER XC2 RNA[16] in 10 μl PIPES-formamide[23,26] at 42 °C for 20 h before digestion with S1 nuclease[26]. S1-resistant DNA fragments were resolved in a 6% (w/v) acrylamide–urea gel; autoradiography was for 4 days with one intensifying screen. Arrow indicates position of a 330-base fragment, corresponding to the size of the full-length probe.

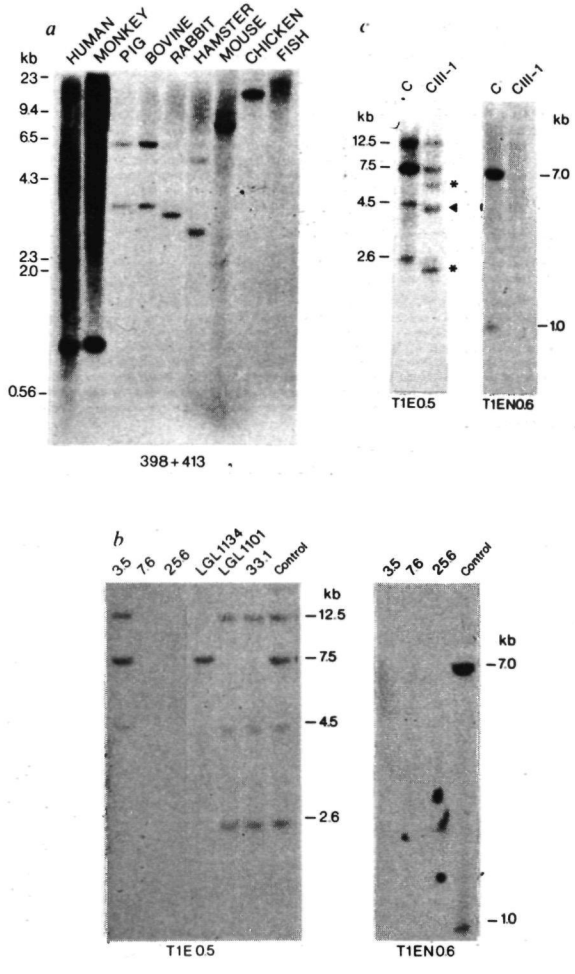


FIG. 3 Southern blot analysis of genomic and cDNA clones. *a*, A mixture of single-copy probes 413 (0.5 kb *Eco*RI) and 398 (1.0 kb *Eco*RI) shows cross-species homology to DNAs of various mammals, and to chicken. *b*, Probes T1E0.5 (segments A and B; see Fig. 1) and T1EN0.6 (segment D and part of segment E; see Fig. 1) detect deletions in male patients with TCD. Deletions in patients 7.6 and 25.6 span all cloned exons from the putative TCD cDNA. In the DNA of patient 3.5, the *Eco*RI bands of 2.6, 1.0/1.6, and 7.0 kb are deleted. The 1.6 kb *Eco*RI fragment contains only 27 bp of segment D, and is therefore not identified with probe T1EN0.6. Deletion LGL1134 spans segments B and C; deletions LGL1101 and 33.1 merely span segment A. *c*, The human–hamster cell hybrid CIII-1 contains the der(13) chromosome from a female with a balanced t(X; 13) translocation associated with TCD[5,15]. This cell line, which contains the Xq21.2-qter part of the human X chromosome, carries all genomic fragments detected by clone T1E0.5, and lacks the two *Eco*RI fragments detected by T1EN0.6. The 4.5-kb *Eco*RI band in control male DNA (control) is represented by a polymorphic variant of 4.2 kb in cell line CIII-1, which comigrates with a hamster band (triangle; F.P.M.C., unpublished results). The extra fragments detected by T1E0.5 in CIII-1 DNA are hamster-specific (see asterisks).

METHODS. Genomic DNAs (10 μg of each) were digested with *Eco*RI. The fragments were subjected to electrophoresis and blotted to GeneScreenPlus (NEN) as described[7]. Isolation and radiolabelling of plasmid inserts was done as described[24]. The blot in *a* was hybridized in 0.5 M NaH_2PO_4 pH6.8, 7% (w/v) SDS, 1 mM EDTA, 50 μg ml^{-1} sonicated, denatured herring sperm DNA, at 50 °C, for 18 h. Washing was in 40 mM NaH_2PO_4, 1% (w/v) SDS at 60 °C for 1 h. The hybridization and washing procedures for *b* and *c* have been published elsewhere[7].

function and no enzymatic active sites could be determined using the PCGene PROSITE program. The C-terminal portion of the protein between residues 253 and 302 carries a so-called PEST region (PEST score of 7.3 according to Rogers *et al.*[17]). Proteins containing one or more PEST regions often exhibit intracellular half lives of less than 2 h, and several of these, such as Fos, Myc and E1A, are important transcriptional regulators[17,18].

Recently, photoreceptor-specific genes have been implicated in autosomal dominant retinitis pigmentosa[19] and two different forms of retinal degeneration of the mouse[20,21]. It is of note, therefore, that the expression of the putative TCD gene is not confined to the eye. This finding is not unprecedented, however. For example, gyrate atrophy, a choroidal disease with clinical similarity to TCD, is caused by a deficiency of an ubiquitously expressed protein, the enzyme ornithine aminotransferase[22]. By analogy, this may indicate that TCD also is due to a generalized

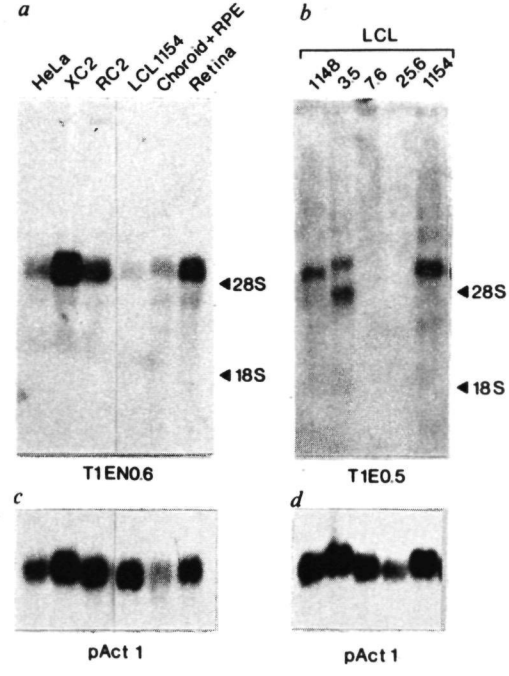


FIG. 4 Northern blot analysis of RNA from several human cell lines and tissues using cDNA clone T1. *a*, Blot containing RNA from HeLa, two retinal cell lines (HER RC2 and HER XC2; ref. 16), an EBV-immortalized lymphoblastoid cell line (LCL1154), as well as human choroid/retinal pigment epithelium and retina. *b*, Blot containing RNA from lymphoblastoid cell lines from patients with different microdeletions encompassing the TCD locus (Fig. 1, and refs 5 and 6), and from two male controls (LCL1148 and LCL1154). *c*, *d*, Hybridization of a hamster actin cDNA clone (pAct-1; ref. 27) to the same blots as in *a* and *b*, respectively. The positions of the 18S and 28S ribosomal RNA bands are indicated.

METHODS. Total cellular RNA was isolated from cells and tissues using the modified LiCl–urea extraction procedure[28,29]. Approximately 10 μg of RNA was dissolved in 30 μl of 50% (v/v) formamide, 2.2 M formaldehyde, 20 mM MOPS, pH 7.0 buffer (4-morpholine-propanesulphonic acid, 5 mM Na-acetate, 1 mM EDTA), loaded onto a 1% agarose gel containing 2.2 M formaldehyde in 20 mM MOPS buffer, and resolved by electrophoresis at 1 V cm^{-1} for 16 h. The gel was rinsed extensively with sterile water for 15 min and RNA blotted to a GeneScreenPlus membrane (NEN) in 10 × SSC. After baking for 2 h at 80 °C, blots were hybridized with random-primed probes. Prehybridization and hybridization was done in 5 × SSPE, 10% (w/v) dextransulphate, 1% (w/v) SDS, and 100 μg ml^{-1} sonicated, denatured herring sperm DNA at 60 °C for 4 and 16 h respectively. Washing was done at the same temperature in 2 × SSC, 1% (w/v) SDS for 40 min.

metabolic defect As this is the first candidate gene for any of the X-linked forms of chorioretinal degeneration, elucidation of its structure and function may provide deeper insight into the molecular mechanisms underlying degenerative processes of the retina, retinal pigment epithelium and choroid. □

Received 4 July; accepted 30 August 1990

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ACKNOWLEDGEMENTS. We thank J. Nathans for the cDNA library; F. S. Collins for the jumping library; A. van der Eb for the retinal cell lines HER XC2 and HER RC2; G. Groeneveld for the genomic library; E.-M. Sankila and F. Brunsmann for DNA of patients with TCD; and F. Oerlemans and L. van Rossum for technical assistance. We also thank L. Pels for eye tissues; J. G. W. Domen for running protein analysis programs; and P. N. Goodfellow for a critical reading of the manuscript. The help of J. A. M. Laurissen and the use of the services and facilities of the Dutch CAOS/CAMM centre is gratefully acknowledged. This work was supported by the Nederlandse organisatie voor Wetenschappelijk Onderzoek, Landelijke Stichting voor Blinden en Slechtzienden, Gelderse Blinden Vereniging, Blinden-Penning and the British Retinitis Pigmentosa Society.

Chapter 10

General discussion and outlook

10.1 Xq21 deletion syndromes: correlation of molecular and clinical features reveals inconsistencies

Characterization of several male-viable duplications and deletions with 52 random DNA probes has enabled us to refine the molecular map of the Xcen-q22 region. Almost all of the deletions spanning part of Xq21 are associated with choroideremia and mental retardation, with deafness being another common feature. Through correlation of clinical and molecular findings, the gene loci for these diseases could be assigned to specific chromosomal intervals, but these studies did not resolve a number of inconsistencies between the genotype and the phenotype of these patients.

Molecular characterization of patients with complex and classical TCD by using anonymous DNA probes (chapter 3) assigned the TCD gene to interval 3 of Xq21 (chapter 3: fig. 1). On the basis of this assignment a likely candidate gene for TCD could be isolated that is expressed in choroid and retina (chapter 9). It is surprising therefore, that clinical symptoms of TCD are lacking in the patient with the largest deletion of all which spans almost the whole Xq21 band (patient RvD; chapter 3). We assume that the absence of ocular signs in this patient is due to his young age of only 10 years at the time of the most recent examination. In TCD, the first characteristic symptom i.e. night blindness, may become apparent between 3 to 40 years of age (Rubin et al. 1966). Though fundus changes may precede clinical symptoms by a number of years, normal fundi have been reported for several young male carriers of the TCD gene (Kärnä 1986).

Deletions spanning interval 2 are associated with different types of deafness. There is one patient (NP) however, who is not deaf in spite of his large deletion encompassing intervals 1 through 6. Thus, it appears that deletion of interval 2 predisposes for, but does not necessarily cause, hearing impairment. Patient XL45 shows congenital mixed, i.e.,

conductive and sensorineural, deafness with stapes fixation, whereas the deafness in patients RvD, XL62 and DM is of the sensorineural type. Whether this clinical variability is due to the presence of more than one gene in interval 2 must await the identification of the underlying gene defect(s). Additional proof for the presence of a deafness gene in this chromosomal region has come from linkage studies performed by Brunner and coworkers (1988) and Wallis and coworkers (1988) employing PGK1 and DXYS1, respectively, as genetic markers. Close linkage was established between both markers and a gene underlying a mixed type of deafness with stapes fixation.

Sex-linked mental retardation (MR) has been found in approximately 1 out of 1000 males (Opitz 1986). Fra(X) accounts for about 40% of XL-MR; 60% of cases can be attributed to non-fra(X) or non-specific MR (Mikkelsen 1987). MR is also a common feature in a large number of clinically complex X-linked syndromes, including those that are associated with TCD and microscopically visible deletions at Xq21. A locus for MR can be assigned to interval 2 and/or interval 3 of the Xq21 band, depending on the interpretation of the clinical findings of patient MBU (Hodgson et al. 1987; chapter 3). Additional information has come from a patient with classical TCD, who showed normal intelligence, in spite of the presence of a deletion extending from interval 2 to interval 5 (chapter 8; patient LGL2905). This finding excludes a locus for MR from interval 3, and narrows its location to the proximal part of interval 2. Linkage analyses, performed by others in large families with MR have provided additional evidence for the presence of a gene for MR in the Xq13-q21 region, but these studies have failed to define the location of the gene(s) more precisely (Sutherland et al. 1987; Chudley et al. 1988; Schwartz et al. 1990; H. Brunner, personal communication).

Alternatively, the variable manifestation of MR and deafness in patients with Xq21 deletions could be due to position effects (Henikoff 1990, and references therein) if both relevant genes are not located within but close to the Xq21 deletions. Their expression might be influenced by changes

in the higher order chromatin structure secondary to the repositioning of inactive chromatin blocks. Also, the expression of genes located near the deletions could be disrupted through the influence of silencers that have been positioned nearby, or by the loss of enhancer elements (Orkin 1990).

10.2 Implications and prospects for diagnosis of TCD

Detection of deletions and point mutations

Employing the candidate TCD cDNA as a probe, we have identified deletions in 10 out of 80 TCD patients (chapter 8 and unpublished results). For several of these deletions, one of the breakpoints has been identified by conventional gel electrophoresis and Southern analysis, and in other deletions the breakpoints could be identified using PFG electrophoresis. In families where these molecular data are available, absolutely reliable carrier detection is possible. In the remaining 70 patients no structural abnormalities could be detected using conventional Southern analysis. Here, diagnostic identification of the underlying mutation will require screening of the entire protein coding region, as well as the transcriptional control regions and the intron-exon boundaries of the TCD gene. Northern blotting experiments have indicated that the expression of the putative TCD gene is not confined to the retina and choroid, but includes, HeLa cells and lymphoblastoid cell lines, albeit at a lower level. This finding will enable us to study some of the specific regulatory features associated with the expression of the gene. Preliminary results employing RNA blotting of lymphoblastoid cell lines from patients with TCD that show no gross structural abnormalities at the DNA level, have indicated that in several patients, there is little or no transcription of the candidate TCD gene. Whether these findings result from promoter mutations influencing the expression of the gene, splice mutations preventing correct transport into the cytoplasm, or mutations altering the stability of the transcript, remains to be

elucidated. In order to identify and characterize small structural mutations, two approaches will be used, depending on the transcriptional pattern. Firstly, for those patients that show transcripts of normal size and abundance in EBV immortalized B-cells, the protein-coding region can be studied after first strand cDNA synthesis and subsequent PCR amplification. These cDNA products can be subjected to the chemical mismatch cleavage procedure (Grompe et al. 1989; Cotton et al. 1989), followed by sequence analysis of the region of interest. Alternatively, the ORF is subcloned into a plasmid vector following amplification and subjected to sequence analysis. Secondly, patients that show no, or aberrant, transcripts in EBV-lines can be investigated for mutations in their genomic DNA by amplification of exons of the gene. For this, all exon-intron boundaries must be subcloned and the sequences flanking the exons must be determined. Following amplification, the PCR products will be rapidly screened employing a recently developed technique based on single-strand conformation polymorphism (SSCP; Orita et al. 1989a,b). Aberrant PCR products will be subcloned into a plasmid vector and studied at the nucleotide level, or sequenced directly.

By employing both approaches simultaneously, it should be possible to identify nearly all mutations underlying the disease. As many patients belong to large families, particularly from Finland and Canada, identification of the underlying mutations should have a considerable impact on diagnosis.

Linkage analysis

Until direct mutation detection is feasible, presymptomatic diagnosis and carrier detection will have to rely on family studies and the use of closely linked markers. Our studies have resulted in the identification of RFLPs which are positioned in, or very close to the candidate TCD gene. pZ172 shows an EcoRI polymorphism at a maximal distance of 250 kb centromeric to the gene; pZ11 shows RFLPs for EcoRI, EcoRV, and MspI

(chapter 6 and unpublished results). One of the MspI alleles is also identified by the 5′ part of the candidate TCD cDNA. Moreover, preliminary data obtained by analysing a recently identified deletion indicates that pZ11 is located inside the candidate TCD gene. In addition, rare PstI restriction patterns have been detected with pJ15 and pJ60 in three English TCD families, but not in approximately 40 normal individuals. pJ15 maps very closely but centromeric to the TCD gene; pJ60 is located in the middle of the gene (F.P.M.C., unpublished data). Deletion analysis has enabled us to order all closely linked polymorpic probes from the Xq21 band that flank the TCD gene: Xcen-pX65H7-pCH1-pZ172-(TCD,pZ11)-pXG7c-pDP34-qter.
pZ11 has been employed in linkage studies in Finnish TCD families; so far no recombination between this marker and the disease gene has been observed (z_{max} = 15.6; E.-M. Sankila, personal communication).

10.3 Outlook: study of structure-function relationships for the candidate TCD gene

As discussed in chapter 9, searches in nucleotide and amino acid data banks have not yet revealed homologies with previously cloned genes, and no conspicuous features like zinc fingers, leucine zippers, or transmembrane segments could be detected so far. However, the polypeptide contains a so-called PEST sequence which is characterized by a high concentration of the amino acids proline, glutamic acid, serine, and threonine, and has been associated with rapid protein turn-over (Rogers et al. 1986). This may indicate that the putative TCD gene product plays a role in the regulation of gene expression.

Future efforts to elucidate the function of the candidate TCD gene, and to obtain formal proof for its identity, will concentrate on the in vitro synthesis of its gene product, and on the generation of specific antibodies (Marston et al. 1986, and references therein). These will enable us to study the tissue-specific expression of this gene, as well as the subcellular distribution of its gene product.

Secondly, we will try to develop a mouse model for TCD by making use of recently developed embryonic stem cell and gene targeting technologies (Smithies et al. 1985; Thomas and Capecchi 1987). Embryological and histochemical studies of chimaeric, heterozygous and hemizygous mutant mice may enable us to gain new insights into the pathogenesis of TCD and related disorders. As a prerequisite for these studies, we have recently cloned part of the murine TCD gene (T.J.R van de Pol et al., unpublished results), the molecular characterization of which is in progress.

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The initial aim of this study was the identification and characterization of deletions associated with X-linked contiguous gene syndromes. Employing approximately 200 random DNA probes from the X-chromosome, several male patients with complex syndromes were investigated, the majority of which showed structural abnormalities in the proximal long arm of the X-chromosome. Both duplications and deletions were characterized by 52 DNA probes, which enabled us to construct a detailed physical map of the Xcen-q22 region (chapter 2). With one exception, the deletions spanning part of Xq21 are associated with TCD and MR, with deafness being another common feature. The gene locus for TCD was assigned to the interval spanning the loci DXS95, DXS165, and DXS233. Genes for X-linked deafness and MR were tentatively assigned to the interval defined by the loci DXS121 and DXS232 (chapter 3). By employing probes from the TCD interval, two submicroscopic deletions were identified in patients with non-syndromic TCD which spanned the DXS165 (p1bD5) locus, but none of the other markers from Xq21 (chapter 4).

The chromosomal region near DXS165 was saturated with new markers employing several cloning strategies simultaneously. Successive chromosomal walking and jumping resulted in the isolation of 5 new markers in the vicinity of DXS165, and the identification of two additional deletions in patients with classical TCD. With these clones we could localize four of the eight deletion endpoints and the breakpoint on the X-chromosome of a female with a de novo X/13 translocation and TCD (chapter 5). In addition, we constructed a lambdaZAP library highly enriched for sequences from the TCD locus by making use of preparative FIGE. From a 625 kb SfiI fragment known to carry (part of) the TCD gene, 7 clones were isolated. One of these clones, pZ11, apart from being located inside the gene, proved to be polymorphic for three enzymes (chapter 6 and unpublished results). Finally, a deletion junction clone was isolated which yielded a new marker that is most likely located telomeric to

the TCD gene (chapter 7).

Employing all new probes from the TCD locus we could identify 8 deletions in 69 patients with TCD, and narrow down the position of part of the TCD gene to a DNA segment of 15-20 kbp (chapter 8). A total of 45 kb of genomic DNA spanning this region was cloned into lambda phage vectors. From this segment, 15 single or low copy sequences were isolated and screened for evolutionary conservation. With two of these DNAs, specific hybridisation signals were obtained with DNAs from several vertebrate species, including chicken. Screening of a human retinal cDNA library resulted in the isolation of eight overlapping cDNA clones, that span 4.5 kb, but do not contain the 5′ and 3′ ends of the cDNA. The corresponding gene is not only expressed in retina, choroid and RPE, and two retinal cell lines, but also in HeLa cells and EBV immortalised B cells. The cDNAs encompass an open reading frame of 948 bp which is structurally altered in 8 TCD patients with deletions and in a female with a balanced translocation involving Xq21. These findings provide strong evidence that we have cloned the gene underlying choroideremia. Neither the nucleotide sequence nor the predicted amino acid sequence of the putative gene product revealed any significant homology to genes or protein sequences in NBRF (december 1989), Swiss-Prot (january 1990) and EMBL (april 1990) databases. No topogenic sequences, no domains with known biological function and no enzymatic active sites could be determined using the PCGene PROSITE program (chapter 9). Elucidation of the function of the putative TCD protein should provide new insights into the molecular mechanisms underlying this disorder and other hereditary retinopathies. Strategies for these types of studies are proposed (chapter 10).

Het primaire doel van deze studie was de identificatie en moleculaire karakterisatie van deleties die geassocieerd zijn met complexe X-gebonden syndromen. Gebruik makend van ongeveer 200 DNA sequenties die willekeurig over het X-chromosoom verspreid liggen, werden diverse mannelijke patienten met complexe syndromen bestudeerd. De meeste hiervan vertoonden structurele afwijkingen in het proximale gedeelte van de lange arm van het X-chromosoom. Interstitiële duplicaties en deleties werden gekarakteriseerd m.b.v. 52 DNA sequenties, waardoor wij in staat waren een nauwkeurige fysische kaart van het Xcen-q22 gebied te construeren (hoofdstuk 2). Het fenotype geassocieerd met Xq21 deleties bestaat (met één uitzondering) uit TCD en MR, en dikwijls ook uit doofheid. Het gen locus voor TCD werd toegewezen aan interval 3, hetgeen de loci DXS95, DXS165, en DXS233 omvat. Genen voor X-gebonden doofheid en MR werden voorlopig toegewezen aan interval 2, gedefinieerd door de loci DXS121 en DXS232 (hoofdstuk 3). Gebruik makend van DNA sequenties van interval 3 konden twee submicroscopische deleties gevonden worden in patienten met niet-syndromische TCD. Deze deleties omvatten het DXS165 (p1bD5) locus, maar geen van de andere markers van Xq21 omvatten (hoofdstuk 4).

Het chromosomale gebied rondom p1bD5 werd verzadigd met nieuwe DNA markers door gebruik te maken van diverse kloneringstechnieken. Chromosoom walking en jumping resulteerde in de isolatie van 5 nieuwe markers nabij p1bD5, en de identificatie van twee additionele deleties in patienten met klassieke TCD. Met deze DNA sequenties konden wij 4 van de 8 deletie-breekpunten en een breekpunt op het X-chromosoom van een vrouw met een de novo X/13 translokatie en TCD lokaliseren (hoofdstuk 5). Verder hebben wij een lambdaZAP DNA bibliotheek geconstrueerd die sterk verrijkt was voor sequenties van het TCD locus door gebruik te maken van preparatieve FIGE. Van een 625 kb SfiI fragment, waarvan bekend was dat een deel van het TCD gen zich er op moest bevinden, werden 7 DNA sequenties geisoleerd. Een van deze sequenties, pZ11, bleek niet alleen

gelokaliseerd te zijn in het TCD gen, maar vertoonde ook RFLPs m.b.v. drie enzymen (hoofdstuk 6 en niet gepubliceerde resultaten). Tenslotte werd er een DNA fragment geisoleerd die sequenties bevat welke flankeren aan een microdeletie, hetgeen een nieuwe DNA marker aan de telomere zijde van het gen opleverde. Door vervolgens gebruik te maken van alle nieuwe DNA sequenties van het TCD locus hebben wij 8 deleties gevonden in 69 patienten met TCD, en kon de positie van een deel van het gen gelokaliseerd worden binnen een DNA segment van 15-20 kb (hoofdstuk 8).

Een gebied van 45 kb genomisch DNA die dit segment bevat werd gekloneerd in faag lambda vectoren. Van dit segment werden in totaal 15 unieke of laag-repetitieve sequenties geisoleerd en getest op evolutionaire conservering. Met twee van deze sequenties werden specifieke hybridisatie signalen verkregen met DNAs van diverse vertebraten, waaronder de kip. Screening van een humane cDNA bibliotheek resulteerde in de isolatie van 8 overlappende cDNAs die samen een consensus vormen van 4.5 kb. Dit stuk bevat echter niet de uiterste 5′ en 3′ delen van het cDNA. Het corresponderende gen komt niet alleen tot expressie in retina, choroid en RPE, en twee retinale cellijnen, maar ook in HeLa cellen en EBV geimmortaliseerde B cellen. In de cDNAs zit een open leesraam van 948 baseparen dat structureel veranderd is in 8 TCD patienten met deleties en in een vrouw met een gebalanceerde translokatie met een breekpunt in Xq21. Deze bevindingen maken het zeer aannemelijk dat we het gen betrokken bij TCD gekloneerd hebben. Zowel de nucleotiden als de daarmee corresponderende aminozuur volgorde vertoonden geen significante homologie met genen of eiwitten die verzameld zijn in de NBRF (december 1989), Swiss-Prot (januari 1990) en EMBL (april 1990) databanken. M.b.v. het PCGene PROSITE programma konden geen topogene domeinen, geen domeinen met bekende biologische funkties en geen enzym-actieve posities in het eiwit gevonden worden (hoofdstuk 9). Opheldering van de funktie van het TCD eiwit kan nieuwe inzichten verschaffen in de moleculaire mechanismen die aan chorioderemie en andere erfelijke retinopathieën ten grondslag liggen.

In het menselijke oog worden een drietal lagen onderscheiden die betrokken zijn bij het zien. In één van deze lagen, het netvlies, wordt het licht opgevangen, omgezet in een electrisch stroompje, en doorgegeven naar de hersenen. De andere twee lagen zorgen voor het goed functioneren van het netvlies door o.a. "afvalstoffen" te verwerken en "brandstof" aan te leveren. De drie hierboven genoemde lagen zijn opgebouwd uit hele kleine eenheden, de cellen. Een cel kan men zich voorstellen als een zak met vloeistof waarin zich allerlei eiwitten bevinden. De aard van deze eiwitten, hun plaats en funktie, wordt bepaald door een soort regelcentrum dat zich middenin de cel bevindt. In dit regelcentrum, ook wel de kern genoemd, ligt alle erfelijke informatie opgeslagen in de vorm van DNA, verdeeld over 46 chromosomen. In dit DNA bevinden zich ongeveer 100.000 pakketjes informatie, de zogenaamde genen. Elk pakketje informatie of gen bevat de "blauwdruk" voor een van de eiwitten die zich in de cel bevinden. Indien er met een van de genen iets mis is, kan er sprake zijn van een erfelijke ziekte, want het "defecte" gen kan dan worden doorgegeven aan nakomelingen.

In dit proefschrift staat een erfelijke oogziekte, genaamd choroideremie, centraal. Deze ziekte geeft bij mannen eerst aanleiding tot slecht zien gedurende de nacht, en verergert zich langzaam tot totale blindheid. Toen wij met onze studie begonnen was niet bekend welk gen verantwoordelijk was voor deze ziekte. Voor de meeste erfelijke ziektes is het heel moeilijk te achterhalen, welke van de 100.000 genen defect is. Wij wisten wel dat het gen zich op het X-chromosoom, een van de geslachts-chromosomen, moest bevinden. Ook werd onze speurtocht vergemakkelijkt door de bevinding dat bij een deel van de choroideremie patienten het betrokken gen geheel of gedeeltelijk afwezig was. Door uit normale individuen de ontbrekende stukken gen te identificeren, kon uiteindelijk het "normale" gen relatief snel gevonden en nauwkeurig bestudeerd worden.

Wat is nu het nut van dergelijk onderzoek? De hierboven genoemde oogziekte komt relatief zelden voor, maar maakt deel uit van een grotere groep erfelijke oogziektes waarvan de oorzaken nagenoeg onbekend zijn. De opheldering van de oogziekte choroideremie kan ons dus mogelijk meer inzicht verschaffen in het onstaan van vergelijkbare ziektes. Of dit in de toekomst kan leiden tot een bepaalde vorm van behandeling, is moeilijk te voorspellen. Hiervoor zal nog veel onderzoek met betrekking tot de funktie van het betreffende eiwit nodig zijn. In ieder geval kan er in families waarin deze ziekte voorkomt nu bekeken worden welke personen "drager" zijn van de ziekte, en welke niet. Dit zal de erfelijkheidsadvisering een stuk betrouwbaarder maken. Mijn onderzoek kan tevens een wezenlijke bijdrage leveren aan de fundamentele kennis omtrent tot dusver nog totaal onbegrepen processen die betrokken zijn bij de werking van het netvlies en vaatvlies van het oog.

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CURRICULUM VITAE

Frans Cremers werd geboren op 17 maart 1960 in Etzenrade nabij Jabeek (Limburg). In 1978 behaalde hij het diploma Atheneum-β aan het Bisschoppelijke College te Sittard. In datzelfde jaar begon hij aan een studie Biologie aan de Katholieke Universiteit van Nijmegen. Het kandidaatsexamen (B1g) werd in oktober 1981 afgelegd. In december 1984 werd het doctoraal examen afgelegd met als bijvak Microbiologie (Drs. J.J.A. v. Bruggen, Prof. Dr. G.D. Vogels), en als hoofdvakken Chemische Cytologie (Drs. A.H.M.S.M. van Kuppevelt, Prof. Dr. C.M.A. Kuyper) en Moleculaire Biologie (Drs. J.T. den Dunnen, Prof. Dr. J.G.G. Schoenmakers). Tijdens de doctoraal fase werd het examen Deskundigheid Stralingshygiëne voor een C-laboratorium met goed gevolg afgelegd.

Van januari tot december 1985 was hij werkzaam als wetenschappelijk medewerker bij de Universitaire Transfusie Dienst, werkgroep Transplantatie Serologie van het St. Radboud Ziekenhuis o.l.v. Dr. P. Reekers.

Van januari 1986 tot mei 1990 was hij als wetenschappelijk medewerker verbonden aan de afdeling Anthropogenetica van het St. Radboud Ziekenhuis alwaar het hier beschreven promotie-onderzoek werd uitgevoerd o.l.v. Dr. B. Wieringa en Prof. Dr. H.H. Ropers.

Tijdens het promotie-onderzoek werden vele congressen bezocht, waaronder 'The Ninth and Tenth International Workshops on Human Gene Mapping' te Parijs (1987) en New Haven (1989), de 'Fifth and Sixth World congresses of the International Retinitis Pigmentosa Association' te Melbourne (1988) en Dublin (1990), en de '41st Annual Meeting of The American Society of Human Genetics' te Cincinnati, alwaar hij op 19 oktober 1990 werd onderscheiden met een 'Student Award' in de categorie 'Predoctoral Basic'.
Op 4 februari 1991 werd hij in Essen onderscheiden met de 'Retinitis Pigmentosa Forschungspreis zur Verhütung von Blindheit 1990'.

Sinds juni 1990 is hij werkzaam als Universitair Docent aan de afdeling Anthropogenetica van het St. Radboud Ziekenhuis te Nijmegen. Naast bestudering van de structuur en functie van het TCD gen, legt hij zich toe op de identificatie van andere genen betrokken bij X-gebonden chorioretinale aandoeningen.

Frans Cremers is getrouwd met Diana Buurman en vader van Ruud en Theo.

Stellingen

I

Choroideremie komt als gevolg van Xq21 deleties niet alleen voor als onderdeel van een complex syndroom in combinatie met doofheid en mentale retardatie, maar ook als een geisoleerde aandoening. Dit doet vermoeden dat ook X-gebonden doofheid en mentale retardatie afzonderlijk veroorzaakt kunnen worden door microdeleties in Xq21.

Dit proefschrift.

II

Gezien het ogenschijnlijk gering aantal genen in Xq21 lijkt een complete opheldering van de nucleotidenvolgorde van dit chromosomale segment in het kader van het Humane Genoom analyse project weinig zinvol.

III

De identificatie van mutaties in het staafjespigment rhodopsine in patienten met een autosomale vorm van retinitis pigmentosa maakt het aannemelijk dat mutaties in kegeltjespigmenten eveneens verantwoordelijk kunnen zijn voor retinale dystrofiëen.

IV

In tegenstelling tot de meeste auto-immuunziektes vinden spondyloarthopathiëen hun belangrijkste oorzaak in de expressie van een specifiek HLA-antigen.

Hammer RE, Maika SD, Richardson JA, Tang JP, Taurog JD (1990) Cell **63**, 1099-1112.

V

De conclusies aangaande de identificatie van glyceringsplaatsen in α-, β-, en γ-crystallines van lenzen afkomstig van diabete ratten lijken ongefundeerd aangezien de getoonde aminozuur samenstellingen van de peptides identiek blijken te zijn met runder-, i.p.v. met ratte α-, β-, en γ-crystallines.

Abraham EC, Perry RE, Abraham A, Swamy MS (1991) Exp. Eye Res. **52**, 107-112.

VI

Door hun snelle opeenvolging en door ondeskundig gebruik schieten nieuwe, "verbeterde", versies van tekstverwerkingsprogramma's hun doel vaak voorbij.

VII

Om te voorkomen dat belangrijke berichten verloren gaan tussen de talrijke faxen kan men ze beter per post versturen.

VIII

Een algemeen verbod op het gebruik van de auto voor woon-werk verkeer binnen een afstand van 10 km zou zowel de gezondheid van de mens als het milieu dienen.

IX

De materialen- en middelenbudgetten ten behoeve van moleculair genetisch onderzoek ontwikkelen zich omgekeerd evenredig aan de gestegen kosten.

Nijmegen, 18 april 1991
Frans Cremers